EFFECTS OF RESTRICTED SPECTRAL REARING ON THE DEVELOPMENT OF ZEBRAFISH RETINAL PHYSIOLOGY

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Directed by: Joseph Bilotta, Ph.D., Elizabeth Lemerise, Ph.D., and Farley Norman, Ph.D.

Department of Psychology Western Kentucky University

Research has shown that rearing in abnormal lighting environments affects both visual behavior and retinal physiology in zebrafish larvae. These studies, however, used only darkness and constant white light as the experimental rearing conditions. The purpose of the present study was to assess the effects on the development of zebrafish retinal physiology of rearing larvae in restricted spectral lighting environments. Larvae were reared in one of seven different lighting environments: cyclic white light (the control group), constant blue light, constant green light, constant orange light, cyclic blue light, cyclic green light, and cyclic orange light. Assessment of retinal physiology was done by using the electroretinogram (ERG). The results showed that restricted spectral rearing caused differences in zebrafish retinal physiology. Rearing larvae in any of the constant light conditions caused deficits in sensitivity to ultraviolet and short-wavelength stimuli, but did not cause differences in sensitivity to middle- and long-wavelength stimuli. Rearing larvae in cyclic light also did not cause differences in sensitivity to middle- and long-wavelength stimuli, but did cause extreme deficits in sensitivity to ultraviolet and short-wavelength stimuli in the cyclic green and cyclic orange light rearing conditions. However, the sensitivity of the cyclic blue light rearing group proved to be similar to the control group to stimuli of all wavelengths. It seems that cyclic short-

wavelength light is necessary for proper retinal development. This study provides further evidence supporting the notion that the zebrafish is a viable model for studying the effects of the lighting environment on visual development.

Chapter 1

Introduction and Purpose

The visual system has been the subject of much research throughout scientific history, leading to many discoveries. This research has also uncovered many new questions that have yet to be answered, due to the fact that the visual system, in all species, is so very complicated. While a description of vision can be reduced to merely explaining that it is the transformation of light from the physical world into a neural signal that is interpreted by the brain, exactly how all of that happens is not fully understood. In addition, it is not fully understood what role environmental factors play in the development of the visual system. This question is obviously an important one in that many visual problems could stem from depriving the system of what it needs to develop properly, or providing too much whereby sensory damage can occur.

Much of the research on vision is done using animal models. Many questions concerning vision cannot be answered using humans as subjects because of obvious ethical concerns. Animal models have proven to be very useful in that they provide researchers the opportunity to have control in the manipulation of experimental conditions. Most vertebrates share many visual system characteristics with humans. Thus, understanding visual processing and development in other species provides insight about our own visual system and its development. Another reason to use animal models for study is that a particular species may possess a visual system with certain unique

properties that allow for testing hypotheses that might otherwise not be possible.

Studying the visual system provides insight into how visual processing takes place, and it also provides information about neural processing as a whole. The nervous system is divided into two distinct systems - one being the peripheral nervous system (PNS) and the other being the central nervous system (CNS). The retina is part of the CNS, and because of the fact that it is physically separated from the rest of the CNS, its easy-to-access location provides a unique opportunity to study neural processing that other systems do not. Studying the manner in which retinal neurons function allows insight into how other portions of the CNS function as well.

The Vertebrate Retina

The retina is responsible for turning the light that enters the eye from the physical world into a neural code that can be sent to and then interpreted by the brain. The retina contains different neurons that are divided into layers that are generally divided into two types: layers made of cell bodies (nuclear layers) and those made of synaptic connections between cells (plexiform layers; see Dowling, 1987). At the outermost portion of the retina, at the back of the eye, is the outer nuclear layer (ONL), and it is here that the photoreceptors are located. The photoreceptors are divided into two types: rods and cones. The light that comes into the eye passes through all of the other layers of the retina to reach these photoreceptors. The tips of the rods and cones (the outer segments) contain photopigments, which are sensitive to light. The photopigments convert the light stimulus that has reached them into electrical signals. The inner nuclear layer (INL), which is situated just inside of the ONL, contains the cell bodies of horizontal cells, bipolar cells,

and amacrine cells. The third nuclear layer, the ganglion cell layer, consists of the cell bodies of ganglion cells. It is the axons of these ganglion cells that combine to form the optic nerve, which transfers the visual information to the brain. There are two plexiform layers: the inner plexiform layer (IPL), which is made up of the synaptic connections between bipolar, amacrine and ganglion cells, and the outer plexiform layer (OPL), which is made up of the synaptic connections between photoreceptors, bipolar and horizontal cells (Dowling, 1987).

The Electroretinogram

As a neural message passes from one layer of the retina to the next, the different layers of the retina produce unique electrical potentials. By using the electroretinogram (ERG), which is a massed electrical potential, the electrical potentials of the various retinal neurons can be measured. The ERG consists of separate components that have been found to correspond to the responses of the different retinal neurons (see Dowling, 1987). The initial component of the ERG is a voltage-negative response, which is called the a-wave, and corresponds to the electrical activity of the photoreceptors. Following the a-wave is a voltage-positive response called the b-wave, which corresponds primarily to the electrical activity of the ON-bipolar cells. Sometimes at stimulus termination another voltage-positive response called the d-wave is evident. There are a number of possibilities as to where this signal originates. Some believe that it corresponds to the photoreceptors turning off at stimulus termination (Dowling, 1987), and others believe it corresponds with the response of the OFF-bipolar cells (Mills & Sperling, 1990). It is also possible, however, that it is a combination of the two.

Zebrafish as a Visual System Model

A model for vision study. Zebrafish have proven to be an excellent model for the study of visual development, and there are many advantages to using this model (Bilotta & Saszik, 2001). They breed prolifically and reach adulthood in a short period of time, which allows for developmental investigation from hatch to adulthood in a short time frame. The fact that zebrafish have a transparent eggshell allows researchers to study the development of the animal without disturbing the growing environment. An added benefit of the transparent eggshell is that it allows the developing visual system to be exposed to different lighting conditions as early as fertilization, which allows one to study how light exposure at different times in development has an effect on visual development. Zebrafish development has been well documented (Westerfield, 1994), which allows comparisons of experimental subjects with normal subjects to determine where any differences lie. This transparency is a benefit to using the zebrafish over the goldfish, since the eggshell of the goldfish is not as transparent and the developmental timeline of the goldfish is not as well documented as that of the zebrafish.

The zebrafish is a very useful model for studying vision because its visual system is so similar to that of other species. The anatomical development of the retina in zebrafish has been studied (Branchek & Bremiller, 1984; Schmitt & Dowling, 1996) and has been found to be very similar to that of other vertebrates such as the mouse (Cepko, Austin, Yang, Alexiades, & Ezzedine, 1996). Another important characteristic of zebrafish vision is that larvae are able to respond to visual stimuli before their visual systems have fully developed (Branchek & Bremiller, 1984), allowing researchers to

study the relationship between the development of the retina and visual physiology and behavior (Bilotta & Saszik, 2001). Although zebrafish are a good model for vision study because of their similarities to other species, they are also a good model because of their differences. In addition to possessing the three cone types that higher primates such as humans possess, zebrafish also have a fourth cone type for ultraviolet vision (U-cone; Robinson, Schmitt, Harosi, Reece, & Dowling, 1993). Possession of this fourth cone type allows research to be conducted that could not be done with many other animal models.

Zebrafish retinal physiology. As was mentioned, zebrafish do possess four cone types, as studies using microspectrophotometric data have shown (Robinson et al., 1993). A later study done by analyzing spectral sensitivity data found that the adult ERG b-wave does receive contributions from all four of the cone types (Hughes, Saszik, Bilotta, DeMarco, & Patterson, 1998). This study also found evidence that supports the notion that there are opponent interactions between the S- and M-cones (M-S) and between the M- and L-cones (L-M). Opponent mechanisms such as these are believed to be essential for color vision processing.

Although adult zebrafish data show that there are opponent mechanisms between some of the cone types, opponency has not been found in data collected from larvae zebrafish (Saszik, Bilotta, & Givin, 1999). In this study, the spectral sensitivity data found there to be contributions of all four cone types to the ERG b-wave of the larvae. However, even at the age of 24 days postfertilization (dpf), no opponent interactions were found between the S- and M-cones and between the M- and L-cones, as were found in adults, suggesting that larvae zebrafish are not able to process color.

Zebrafish retinal development. Research has been done on the anatomical development of the zebrafish retina. Branchek and Bremiller (1984) found there were five types of photoreceptors in the adult retina, each with its own photopigment and thus different sensitivity to lights across the spectrum. These are the rods, long single cones (short-wavelength sensitive or S-cones), short single cones (ultraviolet sensitive or U-cones), and double cone outer segments (long-wavelength sensitive and middle wavelength sensitive, or L- and M-cones, respectively). In developing larvae, at 2 dpf retinal layering begins to appear, and photoreceptor inner segments are observed. At 2.5 dpf outer segments begin to appear, although the number is very reduced, especially when compared to the total number of receptors. By 4 dpf there are many more outer segments, and multiple photoreceptor types begin to appear. However, it is not until 12 dpf that all photoreceptor types can be identified and the regular cone distribution found across the retina in fish (i.e., the cone mosaic) is complete (Branchek & Bremiller, 1984).

The physiological development of the zebrafish retina has also been studied (Branchek, 1984). The ERG was used to measure the response of developing rods and cones in larvae zebrafish. It was found that there are no responses until 3 dpf, which is to be expected since there are very few outer segments formed until this time. At 8 dpf the ERG b-wave component of the larvae are 2 log units less sensitive than those of adults. The responses continue to become more sensitive as the larvae become older, and by 24 dpf, the responses are very similar to those of adult zebrafish, however they are not identical. These studies have found that normal physiological development corresponds with anatomical development, and that the mere presence of anatomical structures is not

enough for adult-like physiological responses. These findings, along with spectral sensitivity data from the study by Saszik et al. (1999), provide evidence to support the notion that the synaptic connections have not fully developed in the larvae, and that it is the development of these connections that is necessary for complete physiological maturation.

Light Environment Effects

Visual system development has been shown to be dependent upon more than mere genetic instruction. Environmental conditions, such as the lighting environment, are necessary for proper retinal development. Studies show that abnormal lighting conditions can affect retinal development in both lower and higher vertebrates (Abramov & Hainline, 1991). One reason for this effect upon development is that the retina is not fully mature at birth in many species, and some of the developmental process takes place in the outside world – either out of the womb or out of the shell.

An example of an abnormal lighting condition that can cause detrimental effects to the visual system is found in neonatal intensive care units in hospitals (Abramov, Hainline, Turkel, Lemerise, Smith, Gordon, & Petry, 1984). Infants who have been placed in neonatal intensive care units have been found to have a higher incidence of visual problems, including color vision anomalies (Abramov & Hainline, 1991). Obviously, controlled experiments cannot be done on humans to study the effects of abnormal light rearing conditions on visual development. However, controlled studies can be done on animal models to gain insight into this phenomenon.

Studies done with primates have shown that specific wavelengths of light have an

effect on retinal function, even after the retina has fully developed. Harwerth and Sperling (1974) exposed adolescent rhesus monkeys to intense short-, middle-, or long-wavelength light for one to two hours a day for six to ten consecutive days. They found a reduction in ERG sensitivity to stimuli originating from the portion of the spectrum to which the monkeys were intensively exposed. The reduction was not permanent in monkeys presented with either intense middle- or long-wavelength light. However, the monkeys that were exposed to intense short-wavelength light did experience a permanent reduction in sensitivity to short-wavelength stimuli.

The lighting environment during rearing has also been shown to have effects on the ability of tree shrews to distinguish achromatic from chromatic light (Petry & Kelly, 1991). In this study, tree shrews were reared from birth to adulthood in cyclic red light. It was found that shrews reared in red light were much less able to distinguish chromatic light from equally bright achromatic light. The authors concluded that the results suggest that the neural mechanisms that are responsible for chromatic/achromatic discriminations are affected by the restricted light rearing.

Studies similar to the ones listed above have also been done with lower vertebrates. Behavioral, electrophysiological, and anatomical methods have been used to study the effects of different abnormal lighting environments in both goldfish and zebrafish. Anatomical studies using goldfish found that the anatomy of the retina was not affected by rearing the fish in either constant white light or constant darkness (Raymond, Bassi, & Powers, 1988). A similar study done with zebrafish found that rearing subjects in constant white light, constant darkness, or normal cyclic white light caused no

differences in retinal anatomy between groups (Robinson & Dowling, 1994). Interestingly, this study found that albino zebrafish reared in any of the abnormal lighting conditions did experience adverse anatomical effects.

Although anatomical abnormalities have not been found in these studies, behavioral abnormalities have been found. Constant light, as well as constant dark, has been found to adversely affect visual processing. A study done by Powers, Bassi, and Raymond (1988) found that the behavioral spectral sensitivity of goldfish was diminished after being reared in either constant white light or constant darkness, with the greatest deficits being in the subjects reared in constant darkness. However, constant white light has been shown to have the most adverse effect on visual behavior in zebrafish (Bilotta, 2000). Bilotta used the optomotor procedure to test visual acuity and found that larvae zebrafish exposed to constant light from 0-6 dpf had a visual acuity below that of constant dark-reared and normal cyclic light-reared subjects. The visual acuity of larvae reared in constant dark was also below that of the subjects raised under normal conditions, although the difference was small.

A physiological study done by Saszik and Bilotta (1999) found that abnormal light rearing conditions caused deficits in retinal physiology. Using the ERG to measure the responses of the retinal neurons, results showed that constant rearing in light caused more physiological damage than constant rearing in dark, although constant darkness had a detrimental effect as well. It should be mentioned that the subjects recovered from the adverse effects after a short period of time (21-24 dpf). The largest effects were found in sensitivity to the ultraviolet and short-wavelength areas of the spectrum. This finding is

consistent with that found in other species, such as primates (Harwerth & Sperling, 1974). Zebrafish color processing, which appears to be present in adults (Hughes et al., 1998), is not fully developed at hatch (Saszik et al., 1999), and it is apparent from the research done by Saszik and Bilotta (1999) that the environment, particularly the lighting environment, plays a role in visual development.

A number of empirical studies have shown that the lighting environment can play a role in visual development. Developmental theorists have suggested that the environment may play different roles in the development of perceptual capabilities, such as vision (Aslin, 1981; Gottlieb, 1981). The maturation model suggests that perceptual capabilities may develop normally without experience (i.e., independent of the environment). Past research indicates that this model is not valid in visual development. The maintenance model proposes that proper development has a maturational basis, but is maintained by the appropriate experience. The facilitation model states that development is sped up in the presence of experience. Note that in this model the particular function would still appear without the particular experience, although at a slower pace. Attunement is a model that proposes that without experience development is stunted and never reaches its full potential, and only with experience is proper development achieved. The induction model proposes that development cannot take place at all in the absence of experience. This model also has been shown by past research to apply to the development of the visual system (Saszik & Bilotta, 1999). In fact, past research on zebrafish visual development suggests that the only two models that may occur in visual system development are the attunement and facilitation models (Saszik & Bilotta, 1999; Saszik

et al, 1999).

Purpose and Hypothesis

As has been mentioned, the retinal physiology of zebrafish has been studied after rearing them in abnormal lighting conditions (Saszik & Bilotta, 1999). This study, however, looked only at the effects of rearing in either constant white light, or constant darkness. Studies such as the 1999 study by Saszik and bilotta have not looked at the effects of rearing zebrafish in only certain portions of the spectrum as in studies done with higher vertebrates (Petry & Kelly, 1991; Harwerth & Sperling, 1974).

The objective of the current study was to examine how restricted spectral rearing influences visual development. It is known, based on previous work done by Saszik and Bilotta (1999), that white light stimulates all cone types, and that overstimulation with white light causes visual abnormalities. The question now is what effect would selectively stimulating (or selectively overstimulating) certain cone types, while depriving other cone types of stimulation, have on visual development? Would the effects in this case be specific to only certain cone types?

In this study, the ERG was used to assess retinal physiological functioning of larvae exposed to different spectral rearing conditions. Some groups were reared in constant light from a narrow portion of the spectrum. Other groups were reared in cyclic lighting conditions using light with the same spectral properties as those used in the constant light conditions. In addition, a control group was reared in normal cyclic white light. The ERG responses of this control group were compared with those of the other groups to determine how restricted spectral rearing affects retinal development.

It was hypothesized that fish reared in constant spectrally-restricted light environments would show a deficit in sensitivity to the portion of the spectrum in which they were reared. Hypothetically, cone types that are sensitive to this portion of the spectrum should be affected in the same way observed under constant white light rearing conditions, which have been shown to cause deficits in sensitivity. Fish reared in constant spectrally-restricted light environments were also expected to show a deficit in sensitivity to other portions of the spectrum, although not as great. Hypothetically, cone types that are sensitive to these portions of the spectrum should be affected in the same way observed for constant darkness rearing conditions, which have been shown to cause deficits in sensitivity that are not as great as those found in constant white light rearing conditions.

It was also hypothesized that fish reared in cyclic spectrally-restricted lighting conditions would show a slight deficit in sensitivity to the portions of the spectrum in which they are not reared. The deficits in sensitivity to these portions of the spectrum should be the same as those found in fish reared in constant darkness. These fish were not expected, however, to show any deficits in sensitivity to the portion of the spectrum in which they were reared cyclically. Hypothetically, cone types that are sensitive to this portion of the spectrum should be affected in the same way as observed for normal cyclic white light rearing conditions; thus, abnormalities were not expected to be found in the sensitivity of these cone types.

Chapter 2

Method

Participants

The project used larvae zebrafish (*Danio rerio*) that were bred in-house (Bilotta, Saszik, DeLorenzo, & Hardesty, 1999). Adult breeders were obtained from a local pet store. The breeders were maintained in the laboratory colony for at least two weeks prior to use to ensure that they were healthy. Other than the different lighting conditions, larvae were maintained using standard procedures (Westerfield, 1994). In all of the conditions the temperature of the tank water was kept between 28 and 30 deg C.

Adult breeders were kept healthy by providing them with a diet enriched by both tropical fish flake food (Tetramin) and live brine shrimp. Once ready for breeding they were placed in a five-gallon tank, which had been prepared by covering the floor with marbles or by placing the breeders in a mesh plastic net to ensure that the breeders did not consume the eggs once they had been laid. On the morning of fertilization, zero dpf, once the breeders had laid the eggs and they had been fertilized, the adult fish were removed from the breeding tank. The eggs were then siphoned from the bottom of the tank and placed in their appropriate lighting condition within 45 minutes of fertilization. The larvae were reared in 500-ml plastic containers. Approximately fifty eggs were placed into each container, which was filled with water from the breeding tank. All of the

containers for all of the conditions were floated in a five-gallon tank inside a light-tight box. A water heater was placed inside the tank to control the temperature of the water. The heater was covered with Teflon tape to attenuate the small red light inside of it. Measures were taken to ensure that the participants were not exposed to any light other than the experimental rearing light.

Previous work has shown that physical development of the larvae is not affected by rearing them in these plastic containers as opposed to the larger tanks. Saszik (1998) found no differences in visual acuity, eye diameter or body length between larvae that were raised in these plastic containers from 0-9 dpf and those raised in a ten-gallon tanks. After 10 dpf, those fish that were not used for data collection were returned to 10-gallon tanks with normal cyclic lighting (white light, 14 hr on/10 hr off).

Apparatus

Light rearing system. For each of the spectral lighting conditions, the plastic container that contained the fertilized eggs was floated in the tank in such a manner that it was constantly situated beneath the designated lighting system. The light in all of the conditions except the control group was furnished by means of a 6 v LED lighting system (MiracleBeam, Pacoima, CA). The benefit of using LED lights is that they provide lighting with a very narrow portion of the spectrum. Each of the three systems had LED lights that emit either blue, green, or orange light with peak wavelengths of 450, 540 and 620 nm, respectively. Each lighting system was fixed directly above the plastic containers that contained the eggs/larvae. The lights were at a distance of one inch from the water surface and one to three inches from the larvae, depending on their location inside the

container. The average irradiance of the light that reached the water's surface in each of the conditions was approximately 300 $\mu W/cm^2$. For the constant light conditions the light system was kept on for 24 hours a day during experimental rearing.

For the cyclic light conditions, all of the above mentioned conditions were the same, except that the lighting systems were on an electrical timer that turned the lights on for 14 hours of the day and shut them off for 10 hours of the day. During the latter ten hours the participants were in complete darkness. This cycle of 14 hours on and ten hours off was chosen because it is the standard lighting condition for zebrafish maintenance (Westerfield, 1994).

The control group was reared in 500-ml plastic containers in the same five-gallon tanks in which the adult breeders laid them. These larvae were exposed to normal cyclic white light (14 hours on/10 hours off) 4.5 feet below fluorescent lighting (F40/D; Sylvania, Danvers, MA) with an approximate irradiance of 200 $\mu W/cm^2$.

Optical stimuli. A two-channel optical system was used to provide the visual stimuli that were presented to the subjects (for details, see Hughes et al., 1998). One channel presented monochromatic light, while the other presented the background stimulus, which was a broadband (white) light. The monochromatic light channel used a 150-W xenon arc lamp as its light source (Spectral Energy, Westwood, NJ, Model LH 150). The light that emanated from the lamp was collimated using a quartz lens. The light beam then passed through a water bath, which was used to filter infrared light and reduce the overall temperature of the light. The light beam was then focused onto an optical shutter (Uniblitz, Rochester, NY, Model LS6ZM2). The optical shutter was operated by a

shutter driver (Uniblitz, Rochester, NY, Model D122) that was controlled by the laboratory computer. Once the light beam had passed through the shutter, it was once again collimated by a quartz lens. The light beam then passed through a series of interference and neutral density filters, which were used to control stimulus wavelength and irradiance. The light beam then passed through a polka dot beam splitter (Oriel, Stratford, CT, Model 38106) and was then focused onto a 5 mm-diameter liquid light guide (Oriel, Stratford, CT, Model 77556).

The background stimulus, which was provided by the second channel, used a 250-Watt tungsten-halogen bulb (Oriel, Stratford, CT, Model 6334) as its light source. The light leaving the lamp was filtered for infrared light by using an optical filter. The light beam was then collimated and focused onto an optical shutter. Once the light beam had passed through the shutter, it was again collimated and then passed through neutral density filters to control stimulus irradiance. The light beam was then projected onto the polka dot beam splitter (Oriel, Stratford, CT, Model 38106), which combined the light sources coming from both the first and second light channels. Once the light was combined it was then focused onto one end of the liquid light guide via a quartz lens. The other end of the guide was placed in front of the subject's eye.

Interference and neutral density filters were used to control stimulus wavelength and irradiance. The first channel, which provided monochromatic light, used interference filters (Oriel, Stratford, CT, Model 54161 & Andover, Salem, NH, Model FS10-50) with a half-bandwidth of 10 nm, ranging from 320 to 640 nm. This channel used neutral density filters that ranged from 0.5 to 3.0 log units of attenuation, which could be

combined to provide stimulus attenuation ranging from 0.0 to 6.5 log units. The neutral density filters were made of quartz so that ultraviolet light could pass through. The second channel, which provided the background stimulus, used neutral density filters (Reynard, San Clemente, CA, Model 398) to maintain a background irradiance of 5 $\mu W/cm^2$. This background light was used because it has been found to isolate the photopic system by suppressing rod contributions in both adult and larvae zebrafish (Hughes et al, 1998; Saszik et al., 1999).

Recording apparatus. The electrodes that were used to record the ERG response were glass pipettes. The pipettes used measured approximately 10 μm in diameter at the tip. A 36 gauge chlorided silver wire was suspended in a teleost saline solution inside each electrode. An adjustable arm on a magnetic base was used to both hold the reference electrode in place and to keep it from moving once positioned. In order to position the recording electrode with precision a micromanipulator (World Precision Instruments Inc., Sarasota, FL, Model M3301L) was used.

The signals that originated from both the recording and the reference electrodes were differentially amplified by means of an AC amplifier (Grass Instrument Co., W. Warwick, RI, Model P55). The amplified signal coming from the amplifier was then split. One signal was displayed on a digital oscilloscope (Tektronix, Beaverton, OR, Model TDS 340), and the other was recorded by the laboratory computer. A 1 ms data acquisition rate was used.

Procedures

There were seven different light conditions in which larvae were reared: normal

cyclic white light (LD; 14 hr light/10 hr dark), constant blue light (BB; 24 hr blue light), cyclic blue light (BD; 14 hr blue light/10 hr dark), constant green light (GG; 24 hr green light), cyclic green light (GD; 14 hr green light/10 hr dark), constant orange light (OO; 24 hr orange light), and cyclic orange light (OD; 14 hr orange light/10 hr dark). The larvae in all of the conditions were raised in their designated rearing environments for at least six days immediately following fertilization. Subjects were tested between the ages of 6 and 10 dpf and were only exposed to the designated light-rearing condition prior to testing. This age group was chosen because although zebrafish vision at this age is not fully developed, it has been shown to respond in a predictable manner to all portions of the visual spectrum to which fully developed adults respond, although the responses are different than those of adults, (Saszik et al., 1999). No older age groups were tested in this study because it has been shown that zebrafish retinal development returns to normal by 21-24 dpf after having been removed from abnormal lighting conditions (Saszik & Bilotta, 1999).

Once removed from the experimental lighting environment, the subject was anesthetized with a 0.01% dose of tricaine methanesulfonate (MS-222). The subject was then placed onto a piece of tissue that was placed on a piece of flattened cotton moistened with an anesthetic solution (MS-222), all of which was positioned on a petri dish. To absorb any excess water on the body, which could disrupt the electrical signal, a small strip of tissue paper was placed over subject, like a shroud. The petri dish was then placed under a stereomicroscope (World Precision Instruments Inc., Sarasota, FL, Model 14168) which was located inside a Faraday cage. The reference electrode was placed on

the body and the recording electrode was placed on the subject's eye.

After having positioned the electrodes, the liquid light guide was placed in front of the right eye. The broadband background was then turned on, the door to the Faraday cage was closed and the animal was allowed to adapt to the background for five minutes. This assured that the fish were light-adapted before trials began. At this point stimulus presentation began. An ascending method of limits procedure was used during trial administration. Stimulus irradiance at any given wavelength began below threshold and was increased in 0.5 log unit steps until response saturation.

Each trial consisted of ten 500 ms stimulus presentations that were averaged to produce one waveform. There was a 500 ms inter-stimulus interval between each stimulus presentation, as well as a 50 ms baseline period before the first stimulus was presented. To avoid selective chromatic adaptation by any one cone type, it was necessary to stagger the order of stimulus presentations in 40 nm steps. Thus, the final data set for each subject included responses to stimuli from 320 to 640 nm in 20 nm steps (for details, see Hughes et al., 1998; Saszik & Bilotta, 1999; Saszik et al., 1999). All procedures were approved by the IACUC committee at Western Kentucky University on September 17, 2001.

Chapter 3

Results

Analysis of the data consisted of examining the ERG waveform and statistically comparing the spectral sensitivity functions across the various light-rearing conditions. The following sections describe these analyses and are divided into three separate sections: analysis of the control group, experimental groups reared in constant spectrally-restricted lighting conditions, and experimental groups reared in cyclic spectrally-restricted lighting conditions.

Waveform Analysis

Each ERG waveform was subjected to a digital filter designed to minimize 60 Hz noise. The resulting waveforms were averaged across the ten stimulus presentations to form one waveform. This type of analysis enables one to examine such ERG characteristics as response amplitude and response latency. The current waveform analysis concentrated primarily on the a-, b-, and d-wave components of the ERG. Comparing the waveforms provides insight into how the development of different cell classes in the retina is affected by restricted spectral rearing. The cellular origin of the a-wave portion of the ERG is believed to be the photoreceptors. The origin of the b-wave is believed to be the ON-bipolar cells, and the origin of the d-wave is either the OFF-bipolar cells, the photoreceptors, or a combination of the two. Thus, for example, differences

across groups in the b-wave component would indicate a change in ON-bipolar cell development due to the lighting environment. Waveforms from each experimental group were compared with the waveforms of the control group.

Control group. Shown in Figure 1 is a sample ERG waveform from a 6-10 dpf larva reared in normal cyclic white light (LD). Figure 1a is the averaged response to a 400 nm stimulus, and Figure 1b is the averaged response to a 500 nm stimulus. The b-wave component, which is the initial voltage-positive response at stimulus onset (indicated by the raised horizontal bar along the abscissa), is clearly identifiable in both the response to 400 nm as well as the response to 500 nm. The d-wave component, which is the voltage-positive response at stimulus termination, is much less apparent in the response to 400 nm than it is in the response to the 500 nm stimulus. The a-wave component, which is the initial voltage-negative response at stimulus onset, was extremely small, if present at all, in the responses of the control subjects. Overall, the ERG waveforms of this group were very similar to the waveforms of adult light-adapted zebrafish (Hughes et al., 1998).

Constant spectrally-restricted lighting groups. Sample ERG waveforms from a 6-10 dpf larva from the LD group as well as sample ERG waveforms from a 6-10 dpf larva from the BB group are shown in Figure 2. Figures 2a and 2b are the same as shown previously in Figure 1. Figure 2c is the averaged response of a larva reared in the BB condition to a stimulus of the same wavelength (400 nm) and irradiance as the one presented to the control larva in Figure 2a. As can be noted, there are differences between the two responses. The amplitude of the b-wave component of the larva reared in the BB

condition is not quite as large as the response of the control larva. It also can be seen that, unlike in the control larva's response, there is a stronger d-wave apparent at stimulus termination in the response of the BB larva. Figure 2d is the averaged response of a larva reared in the BB condition to a stimulus of the same wavelength (500 nm) and irradiance as the one presented to the control larva in Figure 2b. The response of the BB larva to a 500 nm stimulus is very similar to the response of the control larva. The waveforms from larvae from the other constant spectrally-restricted lighting groups were very similar to the responses of the BB group, differing slightly only in response amplitude, so they are not illustrated here.

Cyclic spectrally-restricted lighting groups. Shown in Figure 3 are sample waveforms from three 6-10 dpf larvae that were each reared in one of the three cyclic spectrally-restricted lighting environments. Figures 3a and 3d are the averaged responses of a larva from the BD group to stimuli of 360 nm and 560 nm, respectively. The waveforms of the responses from larvae from this group are very similar to those of the control group. However, the responses from the larvae reared in the other cyclic spectrally-restricted lighting conditions proved to be very different.

Figures 3b and 3e are the averaged responses of a larva from the GD group to stimuli of 360 nm and 560 nm, respectively, and Figures 3c and 3f are the averaged responses of a larva from the OD group to stimuli of 360 nm and 560 nm, respectively. The responses are to stimuli of the same wavelength and irradiance as the stimuli presented in Figures 3a and 3d (360 nm and 560 nm, respectively). As can be seen in Figures 3b and 3c, there are no b-wave components in the ERG responses to a 360 nm

stimulus from either the GD or OD groups. In fact, b-waves did not consistently appear in the ERG responses until above 420 nm in the GD group, and 440 nm in the OD group. However, a-waves were apparent in these groups – from ultraviolet wavelengths to 420 nm in the GD group, and to 500 nm in the OD group. These two groups were the only groups in which a-waves were apparent, and thus the only groups in which they could be analyzed.

Spectral Sensitivity Analysis

Calculating spectral sensitivity functions involved plotting the sensitivity of the subjects to each stimulus wavelength. Spectral sensitivity functions were calculated for the a-, b- and d-wave components of the ERG response to stimuli ranging from 320 to 640 nm, when the components were apparent. The a-wave amplitude was measured from the baseline response (response prior to stimulus onset) to the first negative peak. The b-wave amplitude was defined from either the baseline response or the initial voltage-positive response following the a-wave to the largest voltage-positive value during stimulus presentation. The d-wave amplitude was defined from the baseline response to the largest voltage-positive value following stimulus termination.

To determine the subject's sensitivity to each stimulus wavelength, the reciprocal of the log stimulus irradiance (quanta/s/cm^2) that produced a criterion response was derived. This derivation was accomplished by examining the log irradiance-log response function, which was calculated by plotting the response amplitude in microvolts at each stimulus irradiance as a function of log stimulus irradiance (Saszik & Bilotta, 1999). The stimulus irradiance that yielded the criterion response was derived by interpolating on the

log irradiance-log response function using linear regression (Hughes et al., 1998). This derivation was done for all wavelengths to produce the spectral sensitivity function. The criterion responses were –20 μV for the a-wave, and 20 μV for the b- and d-waves. Spectral sensitivity functions were calculated for each ERG component where possible.

Once the b-wave, a-wave, and d-wave spectral sensitivity functions were calculated for each condition, their differences, where possible, were compared. This comparison was done statistically by using two-factor mixed design analyses of variance (ANOVAs) to compare the spectral sensitivity functions of each restricted spectral rearing group with the corresponding function of the control group (i.e., the spectral sensitivity of the b-wave of the control group was compared with the b-wave function of the different restricted spectral rearing groups). The between-subjects factor was light-rearing condition and the within-subjects factor was wavelength. Tukey's HSD post-hoc tests were conducted to examine any significant condition by wavelength interactions.

Finally, after calculating relative spectral sensitivities for the seven conditions, a quantitative assessment of the cone contributions to each spectral sensitivity function was performed. In order to conduct the quantitative assessment, a multiple mechanism model was applied to the data. This model has been used by Hughes et al. (1998) to describe the adult zebrafish ERG b-wave response in previous work with increment threshold data. The model takes the following form:

Eq. 1 $$S_\lambda = (k_1 \times A_{1\lambda}) + (k_2 \times A_{2\lambda})$$

S_λ = the sensitivity at wavelength λ

$A_{x\lambda}$ = the absorptance of a cone type x at wavelength λ

k_1 & k_2 = the weights assigned to the cone inputs

In this model, when k_2 is positive, two synergistic or additive components combine to determine sensitivity. In turn, when k_2 is negative, two antagonistic or opponent components combine to decrease overall sensitivity. When k_2 is zero, the response consists of only one cone contribution. The multiple mechanism model can determine the best-fit cone weights over narrow portions of the spectrum at one time instead of examining the contribution of a cone type across the whole spectrum. This model proves to be advantageous, since it is possible for a given cone type to contribute an inhibitory response to stimuli of certain portions of the spectrum, while at the same time providing an excitatory response to stimuli from other portions of the spectrum. In order to determine the portion of the wavelength spectrum that any given mechanism covers, the shape of the spectral sensitivity function is examined. Dips in sensitivity, or "notches," in the function are good indicators of where the different mechanisms are located. In order to obtain zebrafish cone spectra, templates are generated by normalized photocurrent data from the giant danio *(Danio aequipinnatus;* Palacios, Goldsmith, & Bernard, 1996) to the peak wavelengths of zebrafish cone photopigments that were obtained from microspectrophotometric data (Robinson et al., 1993). Nonlinear regression analysis was used to find the best least-squares fit of the model to the data (Press, Teukolsky, Vetterling, & Flannery, 1992).

Past studies have found that the best fitting model for adult data uses four mechanisms: U-only, S-only, M-S, and L-M cones (see Hughes et al., 1998 for details). However, this model has not been found to be the best fitting for spectral sensitivity data

for larvae zebrafish (Saszik et al., 1999). The best fitting model for the larvae data from this study required four mechanisms (U, S, M, and L); however, all were excitatory. Once the multiple-mechanisms model analysis was conducted, cone weights of the different conditions were then compared, providing insight into how abnormal rearing conditions alter cone contributions to the spectral sensitivity function.

Control group. The spectral sensitivity functions of both the b-wave component (closed circles) and the d-wave component (open diamonds) of the ERG responses of the 6-10 dpf larvae from the LD group are shown in Figure 4. In this figure, as well as all following spectral sensitivity function figures, the symbols represent the data, the lines represent the results of the multiple-mechanism model, and the error bars indicate ± 1 standard error of the mean (SEM). The letters next to the function indicate the contribution of that cone type at that portion of the function.

As can be seen, the spectral sensitivity function of the b-wave component of the ERG of this group is dominated by the U-cone, indicating that this group is most sensitive to ultraviolet stimuli. Sensitivity to short-wavelength stimuli is substantially less than sensitivity to ultraviolet, and even less to middle- and long-wavelength stimuli. There is no readily apparent peak in the function other than at the ultraviolet region of the spectrum, and there are no notches in sensitivity indicating any opponent mechanisms.

The spectral sensitivity function of the d-wave component of the ERG responses of this group show that the d-wave is not sensitive at all to ultraviolet stimuli. However, the function is extremely similar to the b-wave function at the middle- and long-wavelength portions of the function, indicating similar sensitivity to middle- and long-

wavelength stimuli. The d-wave function is best modeled with only two mechanisms (indicated by letters with asterisks). One mechanism receives excitatory contributions from both the S- and M-cones, and the other receives an excitatory contribution solely from the L-cones.

Constant spectrally-restricted lighting groups. Shown in Figure 5 is the spectral sensitivity function of the b-wave component of the BB group (closed squares), along with the function of the LD group (closed circles) to allow for comparison. The most apparent characteristic of this function is the drop in sensitivity of this group to ultraviolet stimuli compared to the LD group. It also should be noted that the function of the BB group is very similar to the function of the LD group at the short-, middle-, and long-wavelengths. In this group, as in the LD group, all cone contributions are excitatory, and receive contributions from all four cone types.

The spectral sensitivity functions of the d-wave component of the ERG are not shown for any of the experimental groups. The reason for their absence is due to the fact that the appearance of the d-wave in the ERG responses of these groups was very inconsistent. The analysis of this component of the ERG proved to be incomprehensible at best.

The spectral sensitivity function of the b-wave component of the GG group (closed triangles),along with the function of the LD group (closed circles), are shown in Figure 6. Again, this group is not as sensitive to ultraviolet stimuli as the LD group, although the difference does not appear to be as great as in the BB function. And again, the function of this group is extremely similar to the function of the LD group at the

short-, middle-, and long-wavelength portions of the spectrum, indicating similarities in sensitivity to stimuli of these wavelengths.

The spectral sensitivity function of the b-wave component of the OO group (closed diamonds), along with the function of the LD group (closed circles), are shown in Figure 7. As with the other constant spectrally-restricted lighting groups, it can be seen that the b-wave of this group is less sensitive to ultraviolet stimuli than in the LD group. While it should also be noted that the rest of the function also appears to be less sensitive, it was not significantly so (see below). The indication is that the sensitivity in this group, as in the other constant spectrally-restricted lighting groups, to short-, middle-, and long-wavelength stimuli is similar to that of the control group.

Shown in Figure 8 are the b-wave spectral sensitivity functions of all of the constant spectrally-restricted lighting groups along with the LD group to allow for comparison. Again, what is most apparent is the decline in sensitivity to ultraviolet stimuli of all of these experimental groups. A 4 (control and constant spectrally-restricted rearing conditions – BB, GG, and OO) x 17 (wavelength) mixed design ANOVA was done to compare differences between the constant spectrally-restricted rearing groups and the control group. The ANOVA indicated a statistically significant within-subjects main effect of wavelength, $F(16, 512) = 87.64$, $p < 0.001$, as well as a statistically significant interaction between wavelength and group, $F(48, 512) = 3.44$, $p < 0.001$. There was also a statistically significant between-subjects effect of group, $F(3, 32) = 5.54$, $p < 0.01$. Tukey's HSD post-hoc tests on the group by wavelength interaction revealed that the interaction was due to the differences between the sensitivities of the experimental groups

and that of the control group to the ultraviolet portions of the spectrum. The sensitivities of both the BB and OO groups were significantly lower than the sensitivity of the LD group at all stimuli between 320 and 400 nm ($p < 0.05$). The sensitivities of the GG and LD groups to 380 nm were not significantly different. However, all other responses between 320 and 400 nm were either significantly different or approaching significance ($p < 0.055$). The sensitivities of the groups to stimuli above 400 nm were not significantly different from the sensitivity of the LD group except at 640 nm.

Cyclic spectrally-restricted lighting groups. The b-wave spectral sensitivity functions of both the BD group (open squares), and the LD group (closed circles), are shown in Figure 9. The most striking aspect of these two functions is their similarity. Except for slight differences between sensitivities to middle- and long-wavelength stimuli, the two functions practically overlap each other.

Figure 10 shows the b-wave spectral sensitivity functions of both the GD group (open triangles) and the LD group (closed circles). The a-wave spectral sensitivity function of the GD group is shown in this figure as well (open squares). There were no b-waves in the responses to stimuli shorter than 420 nm, which is why the b-wave spectral sensitivity function starts at 420 nm. The b-wave function is very similar to that of the LD group, when comparing the two functions between 420 and 640 nm. The a-wave was apparent at shorter wavelengths in the GD group, and the spectral sensitivity of the a-wave of this group is very similar to that of the b-wave of the LD group. In fact, the a-wave function combined with the b-wave function of the GD group forms a function that is very similar to the b-wave function of the LD group.

The b-wave spectral sensitivity of both the OD group (open diamonds) and the LD group (closed circles) are shown in Figure 11. As in the previous figure, the a-wave spectral sensitivity function of the OD group (open squares) is shown in this function as well. Like the GD group, there were no apparent b-waves below 440 nm for the OD group, and the a-wave was apparent at shorter wavelengths. Again, if both the a-wave function and b-wave function were combined, they would form a function that is very similar to the b-wave function of the LD group.

All of the b-wave spectral sensitivity functions of the cyclic spectrally-restricted lighting groups are shown together in Figure 12 for comparison. The most interesting aspects of this figure are the absence of any data below 420 nm for both the GD and OD groups, and the striking resemblance of the BD function to that of the LD group. Due to the lack of GD and OD b-waves at the shorter wavelengths, it was necessary to conduct two separate ANOVAs for these four groups. A 2 (control and the BD group) x 17 (wavelength) mixed design ANOVA was done to compare differences between the BD group and the control group. There was a significant within-subjects main effect of wavelength, $F(16, 176) = 38.45$, $p < 0.001$. There was not, however, a statistically significant within-subjects interaction between wavelength and group, nor was there a statistically significant between-subjects effect of group, indicating that the spectral sensitivities of the BD and LD group are very similar. The significant main effect of wavelength merely indicates that there are differences across stimulus wavelength for all of the groups.

A 3 (control, GD group, and OD group) x 11 (wavelength; 440-640 nm) mixed

design ANOVA was done to compare differences between the GD, OD, and control groups. There was a significant within-subjects main effect of wavelength, $F(10, 176) = 15.02$, $p < 0.01$. There was not, however, a statistically significant within-subjects interaction between wavelength and group, nor was there a statistically significant between-subjects effect of group. Again, the significant main effect of wavelength merely indicates that there are differences across stimulus wavelength for all of the groups.

Because the a-waves of the GD and OD groups were apparent, the spectral sensitivity functions of the a-waves of these groups were statistically compared with the b-wave function of the control group by using a 3 (control b-wave, GD a-wave, and OD a-wave) x 7 (wavelength; 320-440 nm) mixed design ANOVA. There was a statistically significant within-subjects main effect of wavelength, $F(6, 120) = 16.91$, $p < 0.01$. There was not, however, a statistically significant within-subjects interaction between wavelength and group. A statistically significant between-subjects effect of group was found, although the Tukey's HSD post-hoc test revealed that this was due to differences between the a-wave functions of the two experimental groups, and not between the a-wave functions of the experimental groups and the b-wave function of the control group. It appears that the a-wave spectral sensitivity functions of the GD and OD are not significantly different than the b-wave function of the LD group.

Model Results and Summary

In summary, bar graphs of the relative cone weights obtained from the multiple mechanism models for the b-wave are shown in Figure 13. The weights range from zero to 1.5, and as can be seen in the figure, all of the cone weights in all of the conditions are

positive. The higher the cone weight, the stronger the contribution of that cone type to the ERG response. In all groups, except the GD and OD groups, the b-wave spectral sensitivity received input from all four cone types; the GD and OD groups only received contributions from M- and L-cones.

In Figure 13a, the cone weights of the constant spectrally-restricted lighting groups are compared with those of the control group. It can be seen in the LD group that the most dominant cone contribution is from the U-cones, followed by a slight contribution from the S-cones, and an even slighter contribution from the M-cones and the L-cones. The BB, GG, and OO groups, however, receive much smaller contributions from the U-cones, with the largest contribution being from the U-cones of the GG group, and that weight is roughly only 20% of that of the LD group. Contributions from the other cone types are similar to those of the LD group, with the only substantial difference being that the experimental groups had less of a contribution from the S-cones.

In Figure 13b the cone weights of the cyclic spectrally-restricted lighting groups are compared with those of the control group. The most interesting characteristic of this figure is the large contribution from the U-cones in the BD group, and the complete absence of contribution from the U-cones in the GD, and OD groups. It should also be noted that there is no contribution from the S-cones in the OD group and that contributions from the S-cones in the GD group are substantially smaller than in the LD group. The cone weights of the BD group are very similar to those of the LD group, with the only noticeable difference being the larger input from the U-cones in the BD group. Also worthy of mentioning is the fact that the U-cone contribution appears to be reduced

in all experimental groups except for in the BD group (see Figures 13a and 13b).

Chapter 4

Discussion

The objective of the present study was to discover how restricted spectral rearing affects retinal development, and what type of lighting environment is necessary for proper retinal development. The question asked was what effect would selectively stimulating (or selectively overstimulating) certain cone types, while depriving other cone types of stimulation, have on visual development. It was expected that constant restricted-spectral rearing would cause deficits in sensitivity to the portion of the spectrum in which the zebrafish larvae were reared, and that cyclic restricted-spectral rearing would cause no differences in sensitivity to the portion of the spectrum in which they were reared cyclically. This hypothesis was based on previous work on abnormal light rearing done by Saszik and Bilotta (1999), which showed that constant white light caused deficits in visual sensitivity, particularly in the ultraviolet portion of the spectrum.

The discussion will be divided into two main sections: a discussion of the waveform analysis results and a discussion of the results of the spectral sensitivity analysis. Within each of these sections there will be a discussion of the results of the control group, the constant spectrally-restricted lighting group, and the cyclic spectrally-restricted lighting group. Finally there will be a section dedicated to general conclusions.

ERG Waveforms

Control group. The most important aspect of the waveform analysis of the control group was the similarity between them and what had been found by Saszik and Bilotta (1999). The fact that the waveforms were so similar between the two studies speaks to the fact that the results are valid. The finding of the d-wave being present at the longer wavelengths, but not the shorter ones, is also consistent with findings of Saszik and Bilotta. However, the reason for the d-wave appearing only in ERG responses to longer stimuli is unknown.

Also of importance is how all of the components of the ERG that are present in adult light-adapted zebrafish were found in the ERG of the 6-10 dpf larvae. This outcome is remarkable, particularly when considering the fact that the research on anatomical development done by Branchek and Bremiller (1984) found that it was not until 12 dpf that all photoreceptor types could be identified. Perhaps all photoreceptor types are present by this age, but only in an immature stage that does not allow for easy identification with the light microscope.

Constant spectrally-restricted lighting groups. The waveforms of the BB, GG, and OO groups were very similar to those of the control group. The only noticeable differences were the reduction in response amplitude and the inconsistency of the appearance of the d-wave. The difference in response amplitude was expected – especially in the portion of the spectrum in which the larvae were reared. Most remarkable, however, was how constant spectrally-restricted light rearing seemed to reduce response amplitude only in the responses to the shorter wavelengths, particularly

to ultraviolet light. This type of response was found in all of these groups, independent of the wavelength of light they were reared in, indicating that constant lighting causes deficits in sensitivity to ultraviolet light but not to light of other wavelengths. Perhaps at this age it is only mechanisms that are sensitive to ultraviolet light that are affected by the lighting environment because only they have matured enough to be susceptible to the environment (Branchek & Bremiller, 1984), and thus the differences are seen only in these areas. This notion is supported by Saszik et al. (1999), who found that the ERG of young larvae (i.e., 4-8 dpf) are most sensitive to ultraviolet stimuli.

Cyclic spectrally-restricted lighting groups. The waveforms of the BD, GD, and OD groups proved dissimilar to each other. The BD group had waveforms that were nearly identical to those of the LD group, suggesting that the lighting environment that is necessary for proper development of sensitivity to ultraviolet stimuli must contain cyclic short-wavelength light (speculations as to the reasons for this are mentioned below). The waveforms of this group suggest that all retinal neurons that contribute to the ERG response are present and functional. The GD and OD groups had waveforms that were extremely different – but only in responses to ultraviolet and short-wavelength stimuli.

Both the GD and OD groups did not have b-wave components in their ERG responses to ultraviolet and short-wavelength stimuli. However, even though the b-wave was not present, the a-wave component was present in response to stimuli ranging from ultraviolet stimuli to 420 nm in the GD group and to 500 nm in the OD group. The presence of the a-wave suggests that the photoreceptors are present and responsive. The absence of the b-wave suggests that perhaps either secondary level neurons (particularly

the ON-bipolar cells) have not been formed or are immature, or that the synaptic connections between these neurons have not completely formed, resulting in the absence of the b-wave.

Spectral Sensitivity

Control group. The spectral sensitivity functions based on the b-wave response allowed assessment of the differences in the ON-bipolar cell physiological development. The spectral sensitivity function of the LD group proved to be nearly identical to that of the control group functions obtained in previous zebrafish development studies (Saszik & Bilotta, 1999; Saszik et al., 1999). Again, the indication is that the procedures were similar to those of the previous studies and that the results are accurate. The most noticeable aspect of the spectral sensitivity function of the LD group is the absolute predominance of the sensitivity to ultraviolet stimuli. It is the cone type that is sensitive to this type of stimulus (U-cone) that develops first in the zebrafish (Branchek & Bremiller, 1984), and Robinson et al. (1993) found that the U-cone is the most numerous cone type found in the retina of adult zebrafish. One possible reason for these findings, and for the predominant sensitivity of this cone type, is that zebrafish are surface dwellers, which means they live in an environment rich in ultraviolet light. The fact that zebrafish live in this type of environment may explain why they rely more heavily on ultraviolet stimuli than on other stimuli – for both feeding (from the larvae stage through adulthood) and mating (during adulthood). This explanation is supported by past research that found that small zooplanktivorous fishes that possess an ultraviolet photoreceptor, such as juvenile trout, rely on ultraviolet light for prey search and detection (Browman,

Novales-Flamarique, & Hawryshyn, 1994). Sensitivity in the zebrafish larvae is not nearly as great to short, middle, and long-wavelength stimuli, suggesting that stimuli that fall in this range are perhaps not as important to the survival of young zebrafish.

Another interesting aspect of the LD spectral sensitivity function is the absence of "notches," indicating the lack of opponent mechanisms that are thought to be necessary for color vision, replicating the findings of Saszik et al. (1999). Perhaps larvae zebrafish do not need color vision for survival, and at this stage in their development, it is solely ultraviolet light that is needed to find food.

The d-wave spectral sensitivity function allows insight into the sensitivity of the OFF-bipolar cells to stimulus termination. The appearance of the d-wave at only the longer wavelengths found here replicates results by Saszik et al. (1999). However, spectral sensitivity analysis of the d-wave could not be conducted in that study because of the shorter stimulus duration (200 ms), which caused the d-wave to be somewhat hidden by the b-wave. In the present study, spectral sensitivity analysis of the d-wave was possible because of the lengthening of the stimulus duration to 500 ms. Retinal neurons that contribute to the d-wave are sensitive to the termination of stimuli. It was found that the d-wave function received contributions from S-, M-, and L-cones. The d-wave function was best modeled with only two mechanisms; one mechanism received excitatory contributions from both the S- and M-cones, and the other received an excitatory contribution solely from the L-cones. The function suggests that the sensitivity of retinal neurons that respond to stimulus termination is only similar to the sensitivity of ON-bipolar cells in responses to middle- and long-wavelength stimuli. This finding may

reflect the fact that the M- and L-cone photopigments are found in zebrafish double cones. Thus, if one is present in zebrafish, the other photopigment must be there. Sensitivity of the ON-bipolar cells is higher to short-wavelength stimuli than is the sensitivity of retinal neurons that respond to stimulus termination, and there was no evidence at all of the d-wave in responses to ultraviolet stimuli.

Constant spectrally-restricted lighting groups. When comparing the spectral sensitivity functions of the BB, GG, and OO groups to that of the LD group, there are two very important aspects that must be mentioned. One is the reduction in sensitivity to ultraviolet stimuli in the experimental groups, and the other is the similarity in sensitivity of all of the groups to short-, middle-, and long-wavelength stimuli. These findings suggest that constant light rearing does not affect the development of retinal neurons dedicated to sensing light stimuli that are not ultraviolet. In other words, constant lighting, no matter what type, only affects zebrafish retinal neurons dedicated to sensing ultraviolet light. This finding is supported by the study done by Saszik and Bilotta (1999), in which they found that constant white light reduced spectral sensitivity, especially sensitivity to ultraviolet light. As it turns out, it would not have mattered what portion of white light they had used for the rearing, because the functions would have been nearly identical to the one they found.

It had been expected that the constant rearing groups that were not reared in the blue light (GG and OO) would be only slightly less sensitive to ultraviolet light than the LD group. The slight reduction is sensitivity was expected because the group reared in constant darkness in the study by Saszik and Bilotta (1999) was only slightly less

sensitive to stimuli from this portion of the spectrum. It would seem that the U-cone mechanisms that were not being stimulated in the GG and OO groups would develop in the same fashion as if they had been reared in complete darkness. However, this notion was found not to be the case. Sensitivity of the BB, GG, and OO groups was not significantly different from each other, suggesting that it is constant light that causes the deficits in sensitivity to the ultraviolet stimuli at this age, independent of its spectral properties. The fact that BB, GG, and OO spectral sensitivity are so similar also suggests that the U- and S-cone mechanisms do not develop independently of the environment. That is to say, U- and S-cone mechanism development is dependent upon the light-rearing condition. This dependent development was not the case for the M- and L-cone mechanisms, since they appear to be unaffected by the constant light environment.

Cyclic spectrally-restricted lighting groups. The spectral sensitivity functions of the b-wave component of the cyclic spectrally-restricted lighting groups proved to be very different from each other in one way, yet in another way very similar to each other. The difference between the experimental and control groups was found in the ultraviolet to short-wavelength portion of the function. The BD group was nearly identical to the LD group across the spectrum, while the other two groups (GD and OD) were not similar to the LD group in the ultraviolet to short-wavelength portion of the spectrum. The GG and OO functions differed from the LD function in the complete lack of b-waves in the ERG responses to ultraviolet and short-wavelength stimuli. The similarity was in the middle- and long-wavelength portions of the functions for the three experimental groups. Sensitivity of the b-wave proved to be nearly identical in all the groups to middle- and

long-wavelength stimuli, just as was found in the constant spectrally-restricted lighting groups.

Important inferences can be drawn from these findings. One inference is that for proper retinal development to occur, cyclic short-wavelength light must be present in the environment of the zebrafish larva. Another inference, mentioned earlier, and that is further supported here, is that the lighting environment does not play a role in the development of the portions of the retina that respond to middle- and long-wavelength stimuli (M- and L-cones).

The a-wave spectral sensitivity function allowed assessment of the sensitivity of the photoreceptors. The GG and OO groups were the only groups for which this type of analysis could be conducted because they were the only groups in which the a-wave appeared. The reason the a-wave appeared in these groups was due to the absence of the b-waves; under normal conditions the a-wave is hidden in the b-wave. The sensitivity to ultraviolet and short-wavelength stimuli of the a-wave of the GG and OO groups appeared to be nearly identical of that of the b-wave of the LD group. The appearance of the a-wave suggests that photoreceptor development (U- and S-cones) was not disrupted in these groups, but that perhaps secondary or tertiary level retinal neurons were either absent or not yet fully mature. Perhaps the improper development that takes place in the GG and OO groups is due to the lack of maturation of synaptic connections between secondary retinal neurons and photoreceptors. It is this "fine-tuning" that appears to be necessary for proper retinal functioning, which seems quite possible, since in the development of normally reared zebrafish larvae, all of the retinal neurons are present

long before the spectral sensitivity function of the larvae is identical to that of the adult. This delay in physiological maturation suggests that it is the development of synaptic connections that is still at an immature level (Branchek & Bremiller, 1984; Saszik et al., 1999).

Summary and Conclusions

In summary, the results from the current study support the basic hypotheses that were put forth in the introduction. It was hypothesized that constant light would cause deficits in visual sensitivity and that cyclic light would promote proper visual sensitivity development. However, the results showed that it is only at the ultraviolet and short wavelength portions of the spectrum where these deficits occur. Sensitivity to other portions of the spectrum occurs independently from the type of lighting environment that is present during rearing. As has been mentioned, it appears that U- and S-cone mechanisms' development is dependent in part on the environment.

The results suggest that it is zebrafish sensitivity to ultraviolet and short-wavelength light that is most vulnerable to restricted lighting environments, and that for the proper development of spectral sensitivity to take place, cyclic light containing short-wavelength light is necessary. This finding is supported by past studies that have looked at the effects of the lighting environment on visual function. For example, Harwerth and Sperling (1974) exposed adolescent rhesus monkeys to intense short-, middle-, or long-wavelength light for one to two hours a day for six to ten consecutive days. They found that the only light that caused permanent reduction in sensitivity was the short-wavelength light, which caused deficits in sensitivity to short-wavelength stimuli. Many

more studies have found that short-wavelength light exposure causes more damage than exposure to longer wavelength light. In fact, the term "blue light hazard" was coined due to so many similar findings. Exactly why short-wavelength light causes more damage is not exactly understood. One possible reason is the fact that U- and S-cones are more fragile anatomically than are the other cone types.

The results also suggest that sensitivity to middle- and long-wavelength stimuli is not dependent upon the type of lighting environment present during rearing, nor upon the development of U- and S-cones. These findings could be due to the fact that the M- and L-cones are not yet fully developed until after 12 dpf (Branchek & Bremiller, 1984), and because of that, larvae tested in this study were not yet sensitive enough to be affected by dramatic light-effects (the cutoff age was 10 dpf). To verify whether or not this is the case, it would be necessary to rear the experimental groups for longer periods of time in the lighting conditions. Another possible reason for the fact that the environment did not affect the development of sensitivity to middle- and long-wavelength stimuli is that the development of this type of sensitivity may be genetically predetermined and, thus, not affected by the environment. This possibility is discussed in greater detail below.

When reviewing the results and comparing how the lighting environment affected visual development in the zebrafish larvae with different models that have been proposed by developmental theorists, it is seen that several models are viable (Aslin, 1981; Gottlieb, 1981). Two models could explain development of sensitivity to ultraviolet and short-wavelength stimuli. The facilitation model, which states that development is sped up in the presence of experience (or slowed down by its absence), is one model that could

explain the U- and S-cone mechanism development that was found. Note that in this model the particular function would still appear without the particular experience, although at a later time in development. In this study, the particular function that would appear at a later time would be sensitivity to ultraviolet and short-wavelength stimuli, and the particular experience that would be absent would be exposure to ultraviolet and short-wavelength light (which occurs in the GG, OO, GD, and OD lighting conditions). To verify that development of sensitivity to ultraviolet and short-wavelength stimuli follows this model, it would be necessary to rear groups in the experimental conditions for longer periods of time to observe whether sensitivity to ultraviolet and short-wavelength stimuli is increased after a period of time without experience. If sensitivity did increase, it would support that it is the facilitation model that takes place. Attunement is another model that might explain the development of sensitivity to ultraviolet and short-wavelength stimuli. This model proposes that without experience, development is stunted and never reaches its full potential, but with experience, proper development is achieved. Again, it would be necessary to test older age groups reared for longer periods of time in the experimental conditions to verify whether or not it is this model that fits the development of sensitivity to ultraviolet and short-wavelength stimuli. If sensitivity to ultraviolet and short-wavelength stimuli remained at the same level as that found in the current study, it would suggest that the attunement model fits the development of sensitivity to ultraviolet and short-wavelength stimuli. However, this model would probably prove to not be the most accurate model due to the fact that the zebrafish retina would most likely regenerate after being removed from the experimental conditions, as was found in the study by Saszik and

Bilotta (1999).

Neither of the models mentioned above can explain the development of the sensitivity of the retina to middle- and long-wavelength stimuli. The results of the current study suggest that development of sensitivity to these types of stimuli occurs independently of the type of lighting environment that the larvae are reared, at least up to the age of 10 dpf. This type of development fits the maturation model, in which it is suggested that perceptual capabilities may develop normally without experience (independent of the environment).

This study proved to be valuable in that it provided a valid means of testing the effects of the lighting environment on the development of zebrafish retinal physiology, particularly the effects of restricted spectral lighting environments. It has been shown that the spectral properties of the lighting environment do cause changes in retinal development to larvae in this age group under these lighting conditions, as was reflected in the waveform and spectral sensitivity analysis. However, the effects of the environment were not as straightforward as had been thought before conducting the study. Visual development appears to consist of an intricate balance of predisposition and experience for normal development to occur. And as was mentioned above, further studies must be conducted before further conclusions can be drawn as to exactly how the environment is influencing retinal physiological development.

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Figure Captions

Figure 1. Sample ERG waveforms from a control group larva (6-10 dpf) zebrafish. In Figure 1a, the stimulus wavelength was 400 nm, and in Figure 1b, the stimulus wavelength was 500 nm. The log irradiance for Figure 1a was 12.90 log quanta/second/cm^2; the log irradiance for Figure 1b was 14.00 log quanta/second/cm^2. In each of the figures, stimulus presentation was 500 ms, and the responses were averaged across the ten stimulus presentations. The raised horizontal bar along the abscissa in each figure represents stimulus onset and termination.

Figure 2. Sample ERG waveforms from control and BB group larvae (6-10 dpf) zebrafish. In Figures 2a and 2c, the stimulus wavelength was 400 nm, and in Figures 2b and 2d, the stimulus wavelength was 500 nm. The log irradiance for Figures 2a and 2c was 12.90 log quanta/second/cm^2; the log irradiance for Figures 2b and 2d was 14.00 log quanta/second/cm^2. Other details as in Figure 1.

Figure 3. Sample ERG waveforms from BD, GD, and OD group larvae (6-10 dpf) zebrafish. In Figures 3a, 3b, and 3c, the stimulus wavelength was 360 nm, and in Figures 3d, 3e, and 3f, the stimulus wavelength was 560 nm. The log irradiance for Figures 3a, 3b, and 3c was 13.37 log quanta/second/cm^2; the log irradiance for Figures 3d, 3e, and 3f was 14.78 log quanta/second/cm^2. Other details as in Figure 1.

Figure 4. The b-wave and d-wave spectral sensitivity functions of the LD group (n = 4). The symbols represent the data, and the lines represent the appropriate best-fit model. The error bars represent ± 1 SEM. The closed circles represent the b-wave, and the open diamonds represent the d-wave. Log relative sensitivity is defined as the reciprocal of the

log stimulus irradiance required to produce a criterion response of 20 μV. The letters next to the function indicate the cone contributions to the function; the letters with asterisks indicate the cone contributions for the d-wave function.

Figure 5. The b-wave spectral sensitivity functions of the BB (closed squares, n = 12) and LD (closed circles, n = 4) groups. The lines represent the appropriate best-fit model, and error bars represent ± 1 SEM. Log relative sensitivity is defined as the reciprocal of the log stimulus irradiance required to produce a criterion response of 20 μV. The letters indicate the cone contributions to the function.

Figure 6. The b-wave spectral sensitivity functions of the GG (closed triangles, n = 13) and LD (closed circles, n = 4) groups. The lines represent the appropriate best-fit model, and error bars represent ± 1 SEM. Log relative sensitivity is defined as the reciprocal of the log stimulus irradiance required to produce a criterion response of 20 μV. The letters indicate the cone contributions to the function.

Figure 7. The b-wave spectral sensitivity functions of the OO (closed diamonds, n = 8) and LD (closed circles, n = 4) groups. The lines represent the appropriate best-fit model, and error bars represent ± 1 SEM. Log relative sensitivity is defined as the reciprocal of the log stimulus irradiance required to produce a criterion response of 20 μV. The letters indicate the cone contributions to the function.

Figure 8. The b-wave spectral sensitivity functions of the BB (closed squares, n = 12), GG (closed triangles, n = 13), OO (closed diamonds, n = 8), and LD (closed circles, n = 4) groups. The lines represent the appropriate best-fit model, and error bars represent ± 1 SEM. Log relative sensitivity is defined as the reciprocal of the log stimulus irradiance

required to produce a criterion response of 20 μV. The letters indicate the cone contributions to the function.

Figure 9. The b-wave spectral sensitivity functions of the BD (open squares, n = 9) and LD (closed circles, n = 4) groups. The lines represent the appropriate best-fit model, and error bars represent ± 1 SEM. Log relative sensitivity is defined as the reciprocal of the log stimulus irradiance required to produce a criterion response of 20 μV. The letters indicate the cone contributions to the function.

Figure 10. The b-wave spectral sensitivity functions of the GD (open triangles, n = 8) and LD (closed circles, n = 4) groups. The a-wave spectral sensitivity function of the GD group (open squares, n = 8) is also shown. The lines represent the appropriate best-fit model, and error bars represent ± 1 SEM. Log relative sensitivity is defined as the reciprocal of the log stimulus irradiance required to produce the criterion response. The letters indicate the cone contributions to the function.

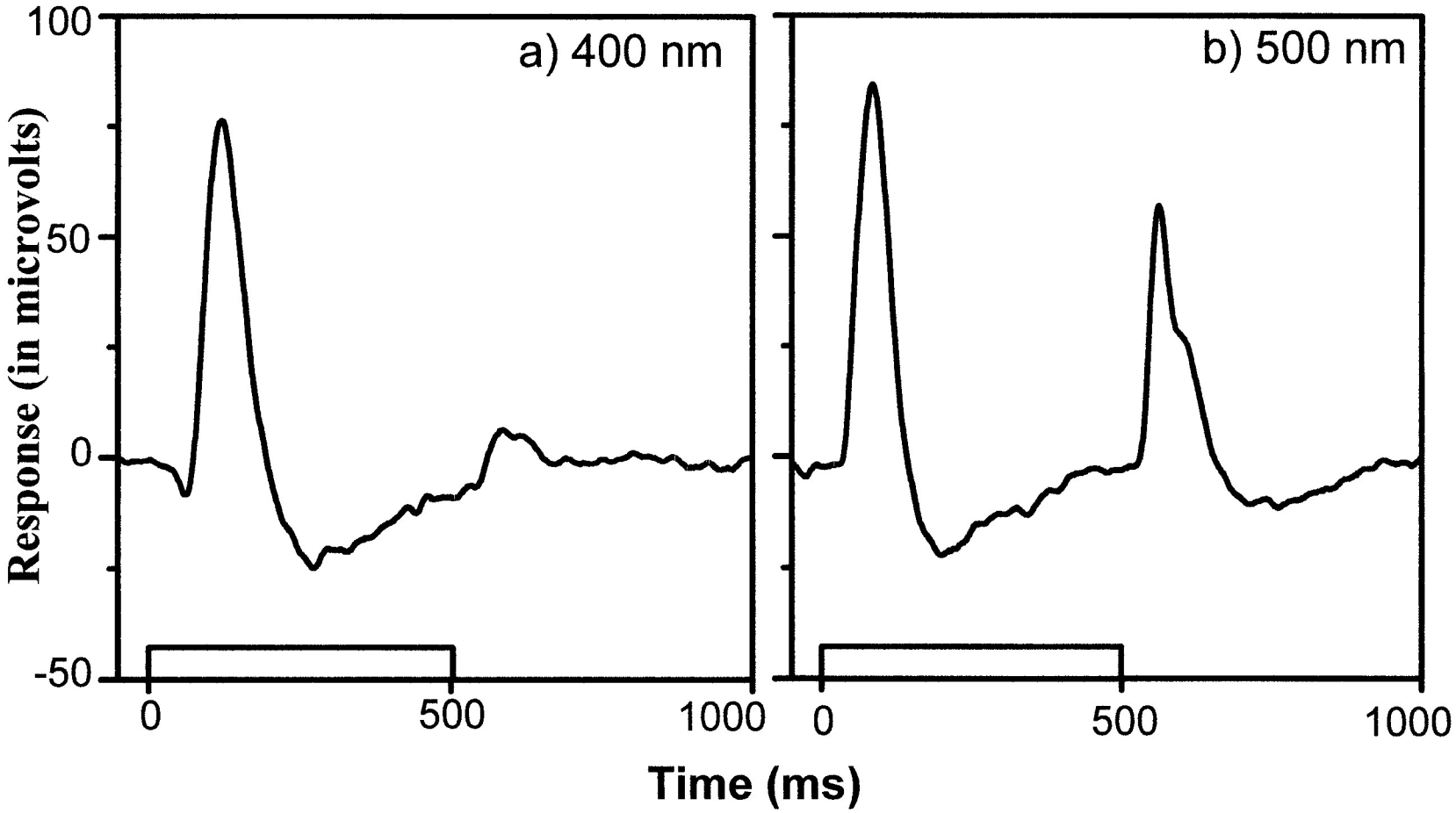
Figure 11. The b-wave spectral sensitivity functions of the OD (open diamonds, n = 8) and LD (closed circles, n = 4) groups. The a-wave spectral sensitivity function of the OD group (open squares, n = 8) is also shown. The lines represent the appropriate best-fit model, and error bars represent ± 1 SEM. Log relative sensitivity is defined as the reciprocal of the log stimulus irradiance required to produce the criterion response. The letters indicate the cone contributions to the function.

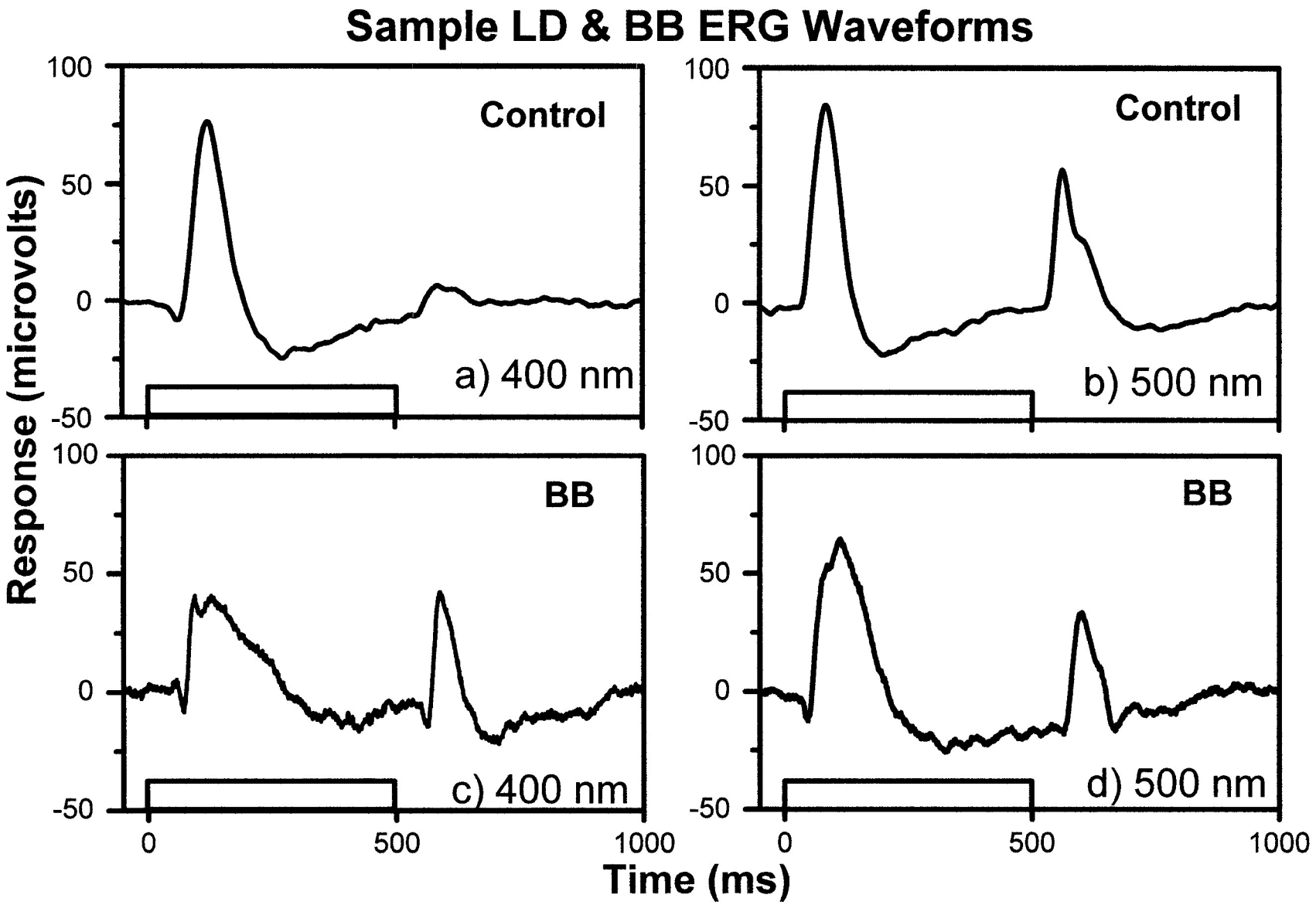
Figure 12. The b-wave spectral sensitivity functions of the BD (open squares, n = 9), GD (open triangles, n = 8), OD (open diamonds, n = 8), and LD (closed circles, n = 4) groups. The lines represent the appropriate best-fit model, and error bars represent ± 1

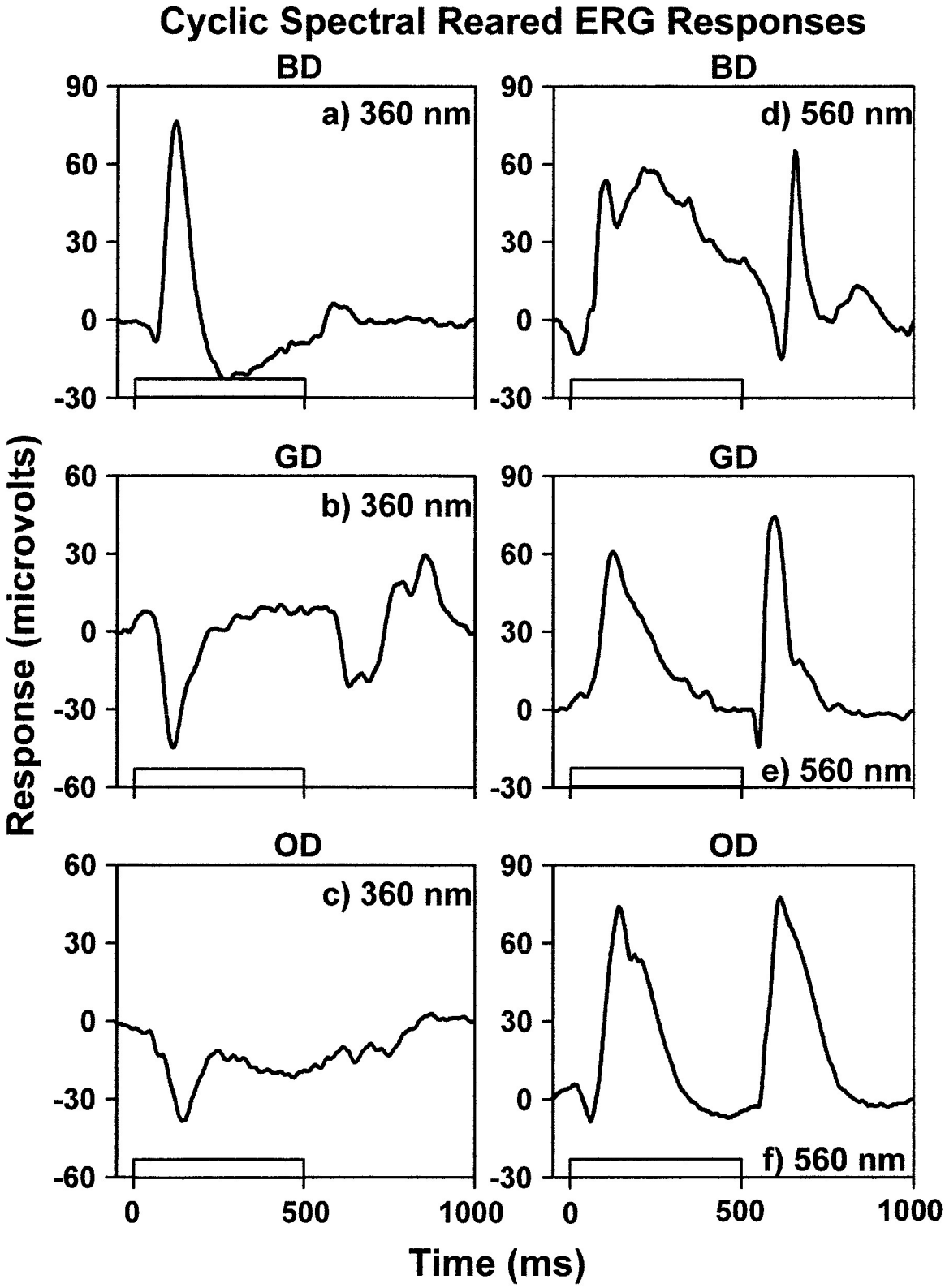
SEM. Log relative sensitivity is defined as the reciprocal of the log stimulus irradiance required to produce a criterion response of 20 μV. The letters indicate the cone contributions to the function.

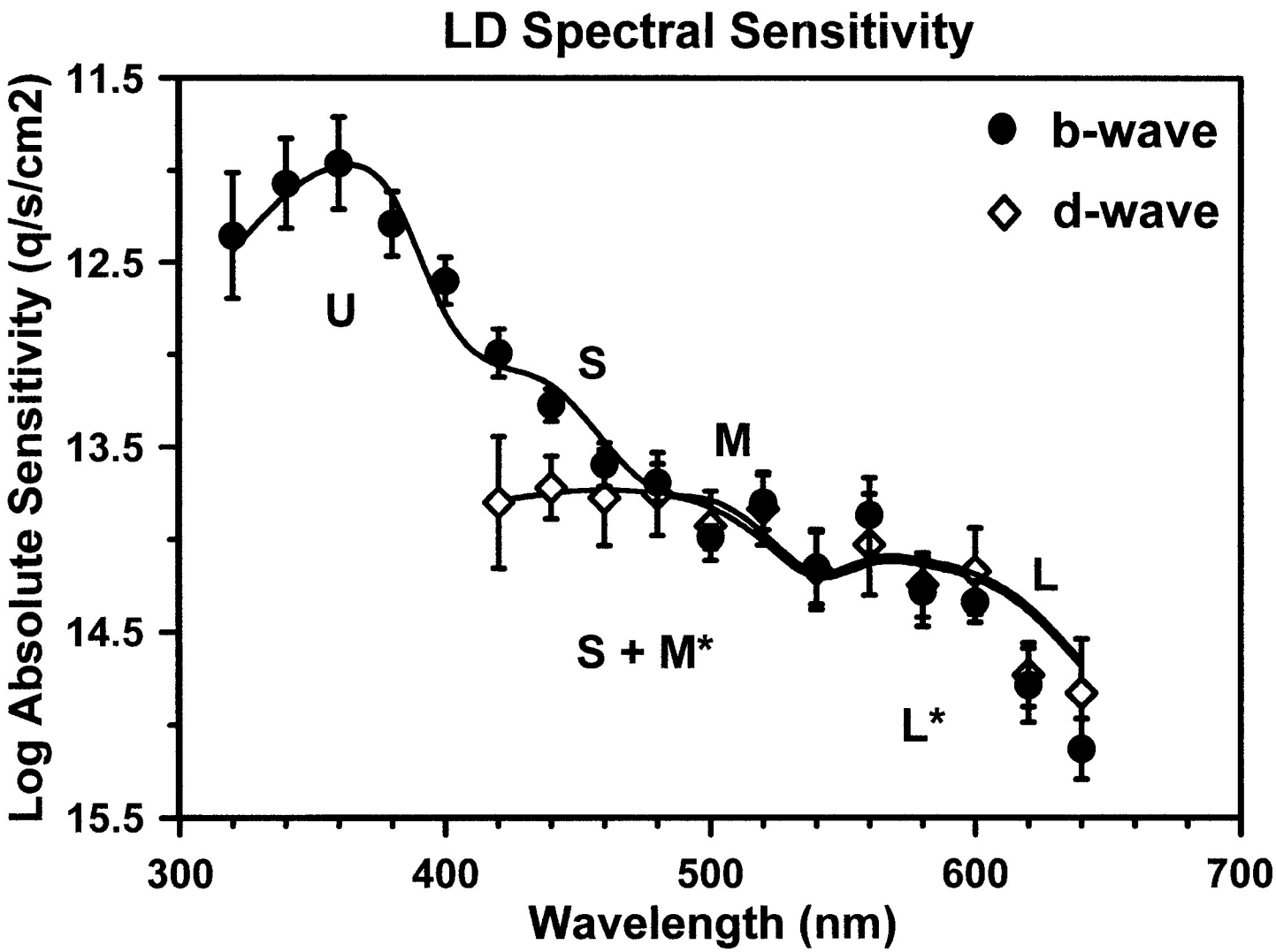
Figure 13. The model weights of the four cone spectra from the best-fit multiple mechanism model. Figure 13a compares the LD, BB, GG, and OO groups, and Figure 13b compares the LD, BD, GD, and OD groups.

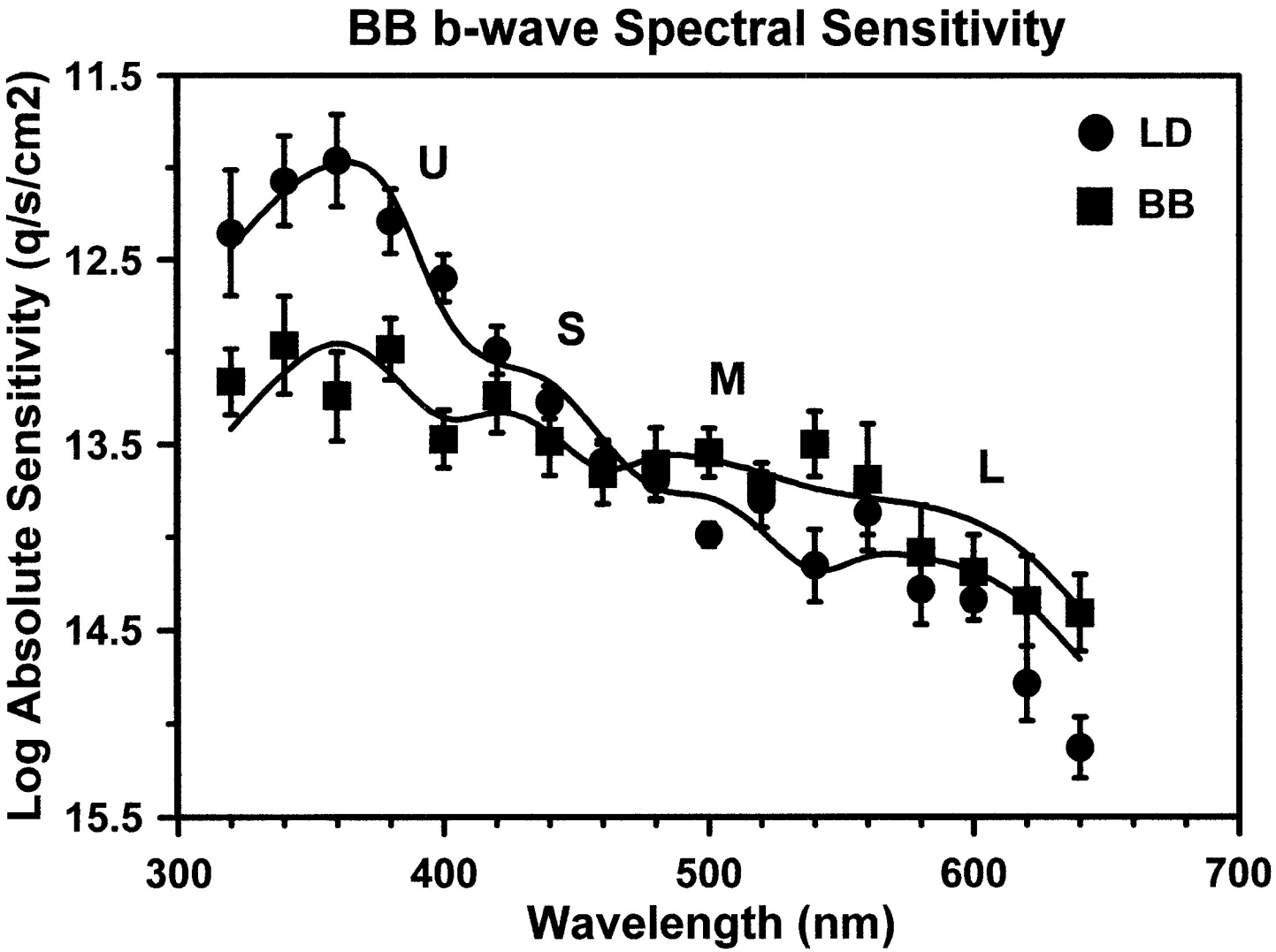
Sample LD ERG Waveforms

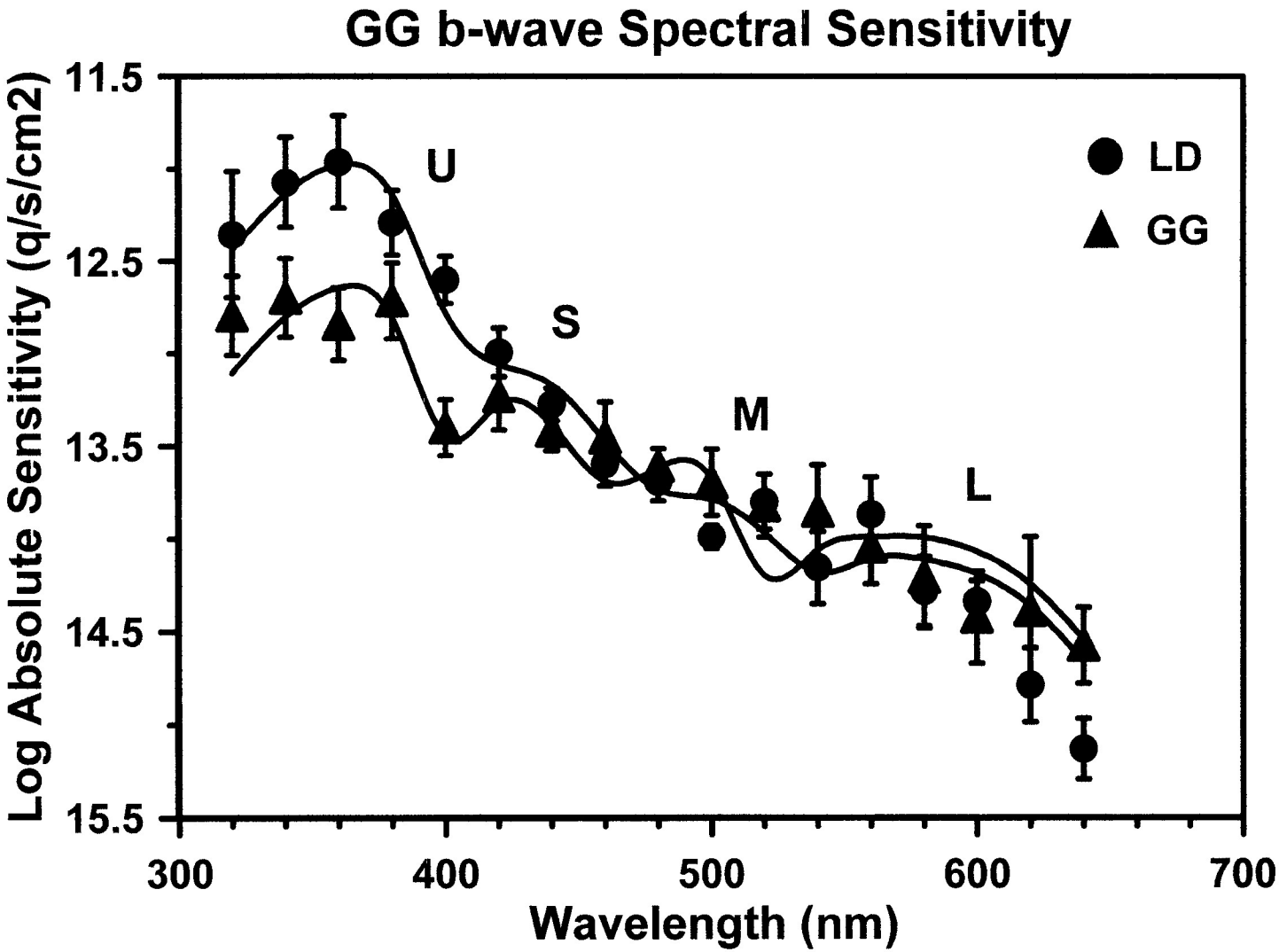


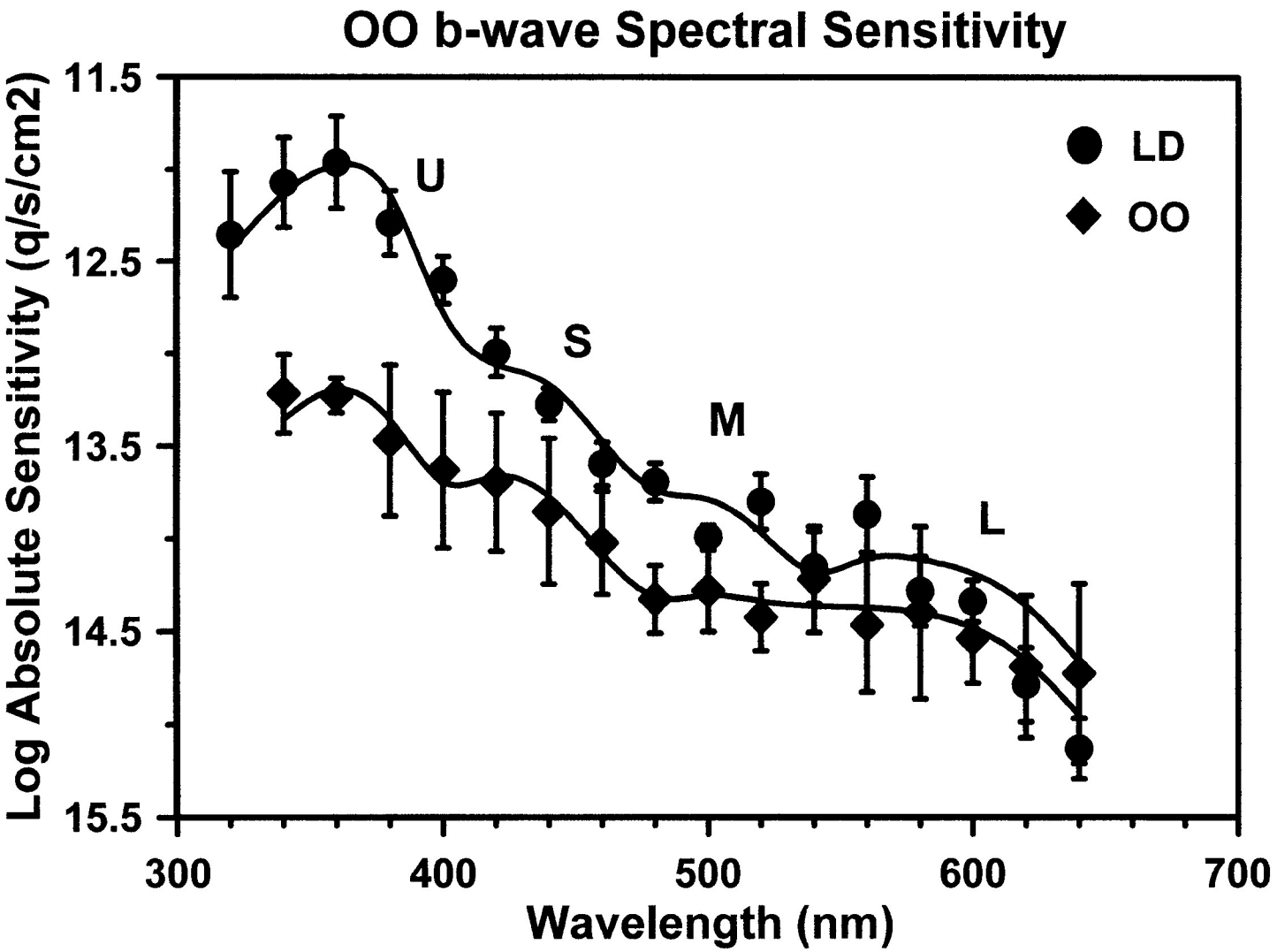

Sample LD & BB ERG Waveforms
Response (microvolts)
100
50
0
-50
Control
a) 400 nm
Control
b) 500 nm
BB
c) 400 nm
BB
d) 500 nm
0
500
1000
Time (ms)

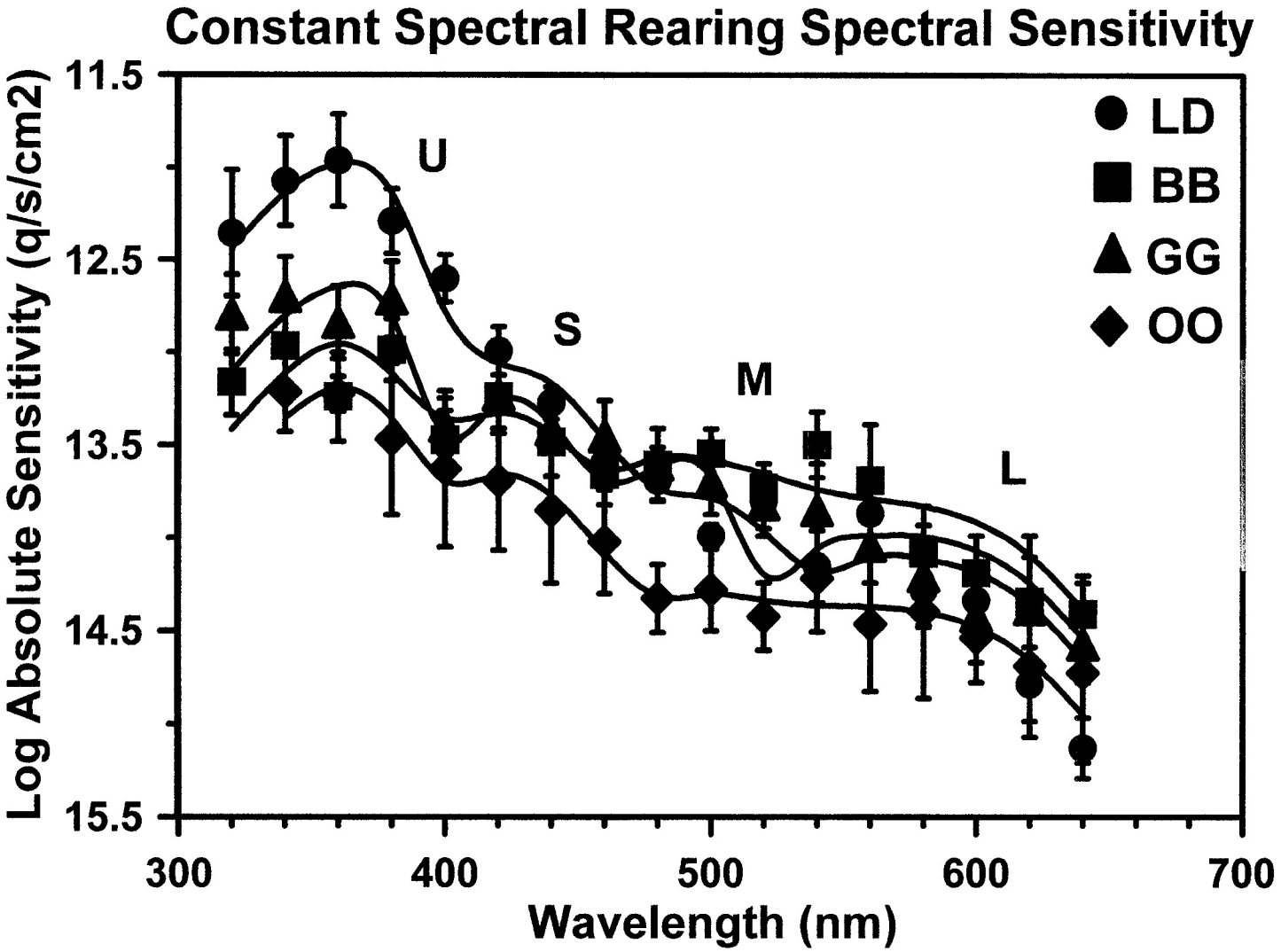

Cyclic Spectral Reared ERG Responses
BD
a) 360 nm
BD
d) 560 nm
GD
b) 360 nm
GD
e) 560 nm
OD
c) 360 nm
OD
f) 560 nm
Response (microvolts)
Time (ms)
90
60
30
0
-30
-60
500
1000

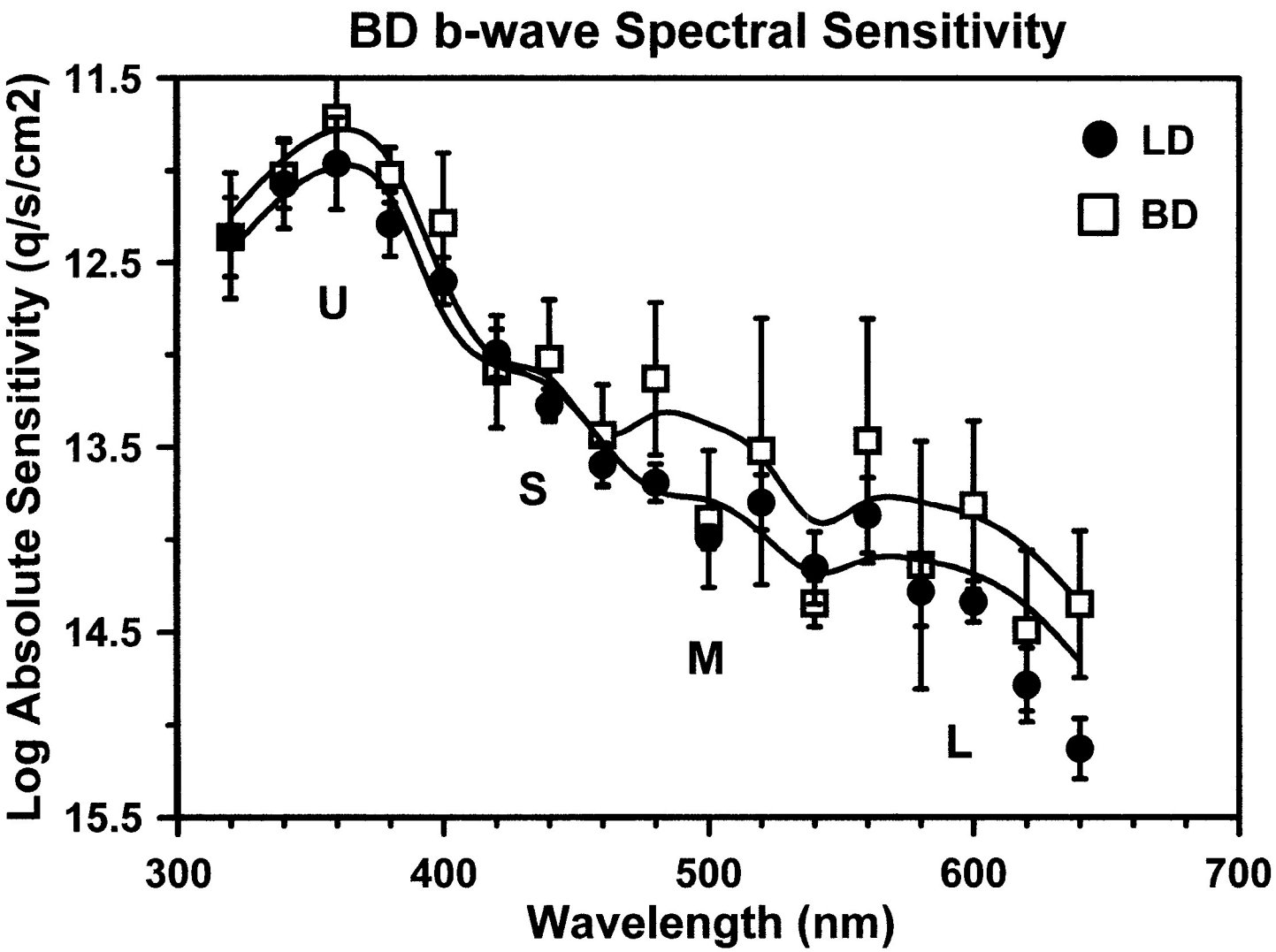

LD Spectral Sensitivity
Log Absolute Sensitivity (q/s/cm2)
11.5
12.5
13.5
14.5
15.5
300
400
500
600
700
Wavelength (nm)
b-wave
d-wave
U
S
M
L
S + M*
L*

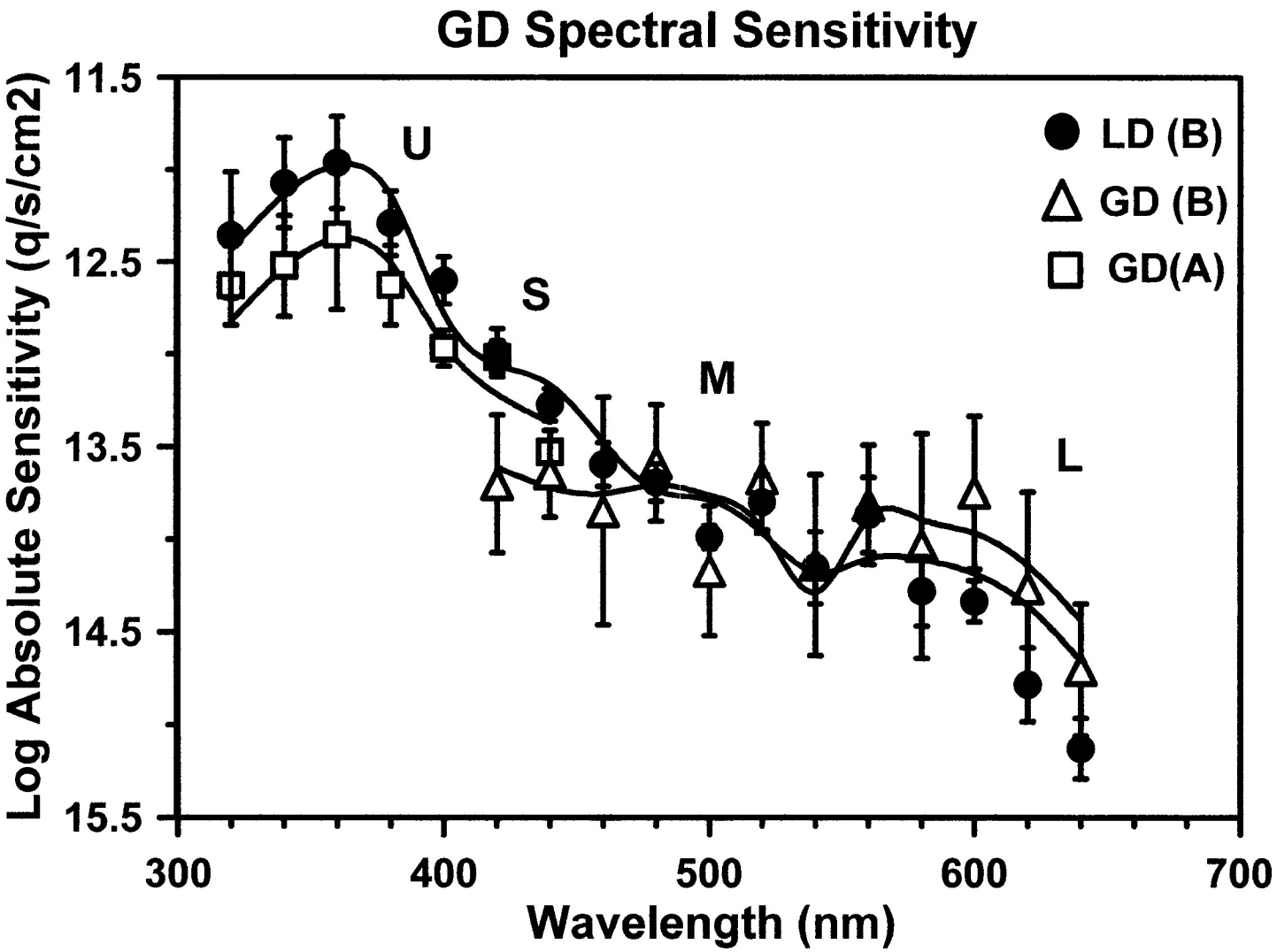

BB b-wave Spectral Sensitivity
Log Absolute Sensitivity (q/s/cm2)
11.5
12.5
13.5
14.5
15.5
300
400
500
600
700
Wavelength (nm)
LD
BB
U
S
M
L

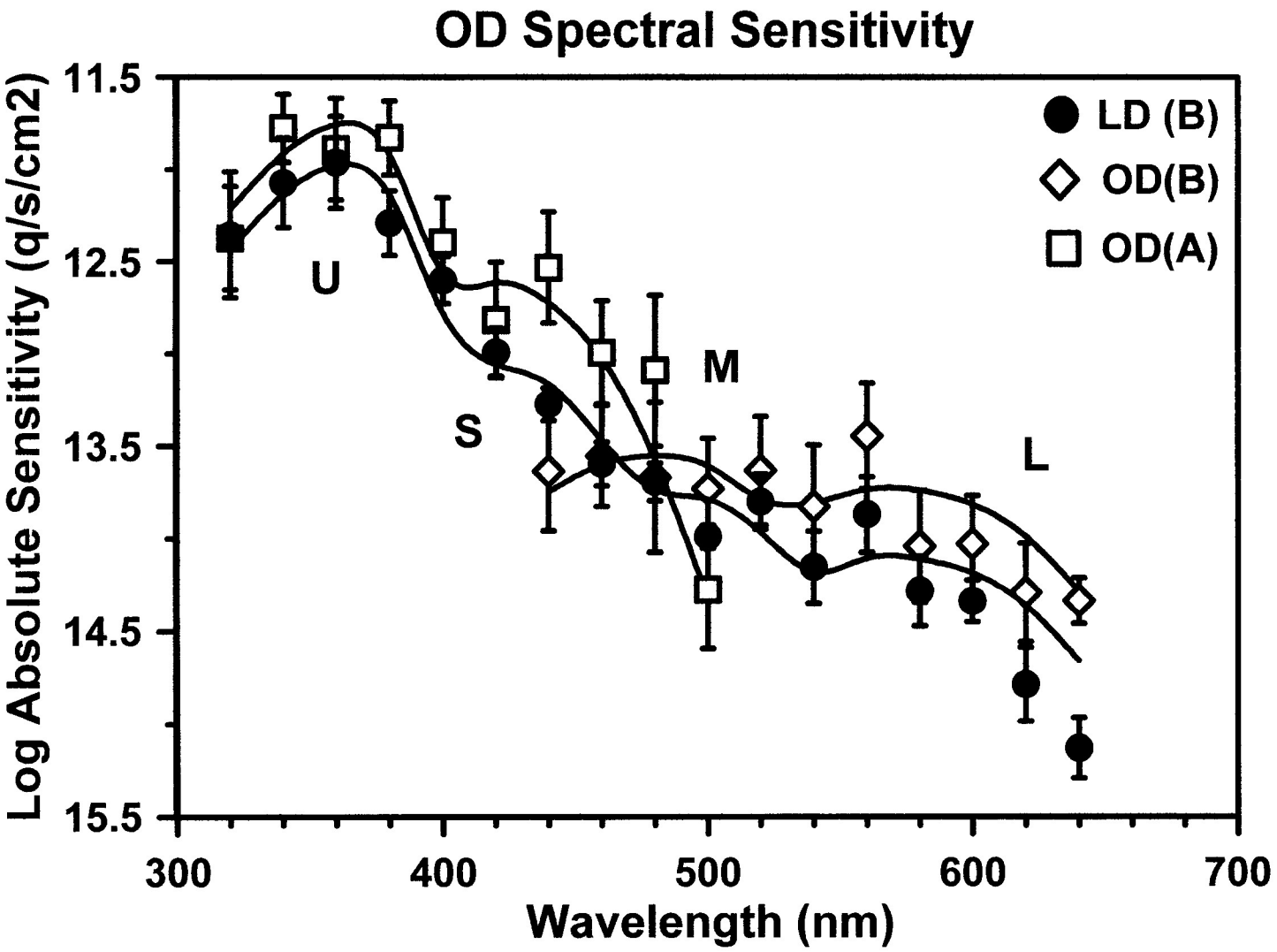

GG b-wave Spectral Sensitivity
Log Absolute Sensitivity (q/s/cm2)
11.5
12.5
13.5
14.5
15.5
300
400
500
600
700
Wavelength (nm)
U
S
M
L
LD
GG

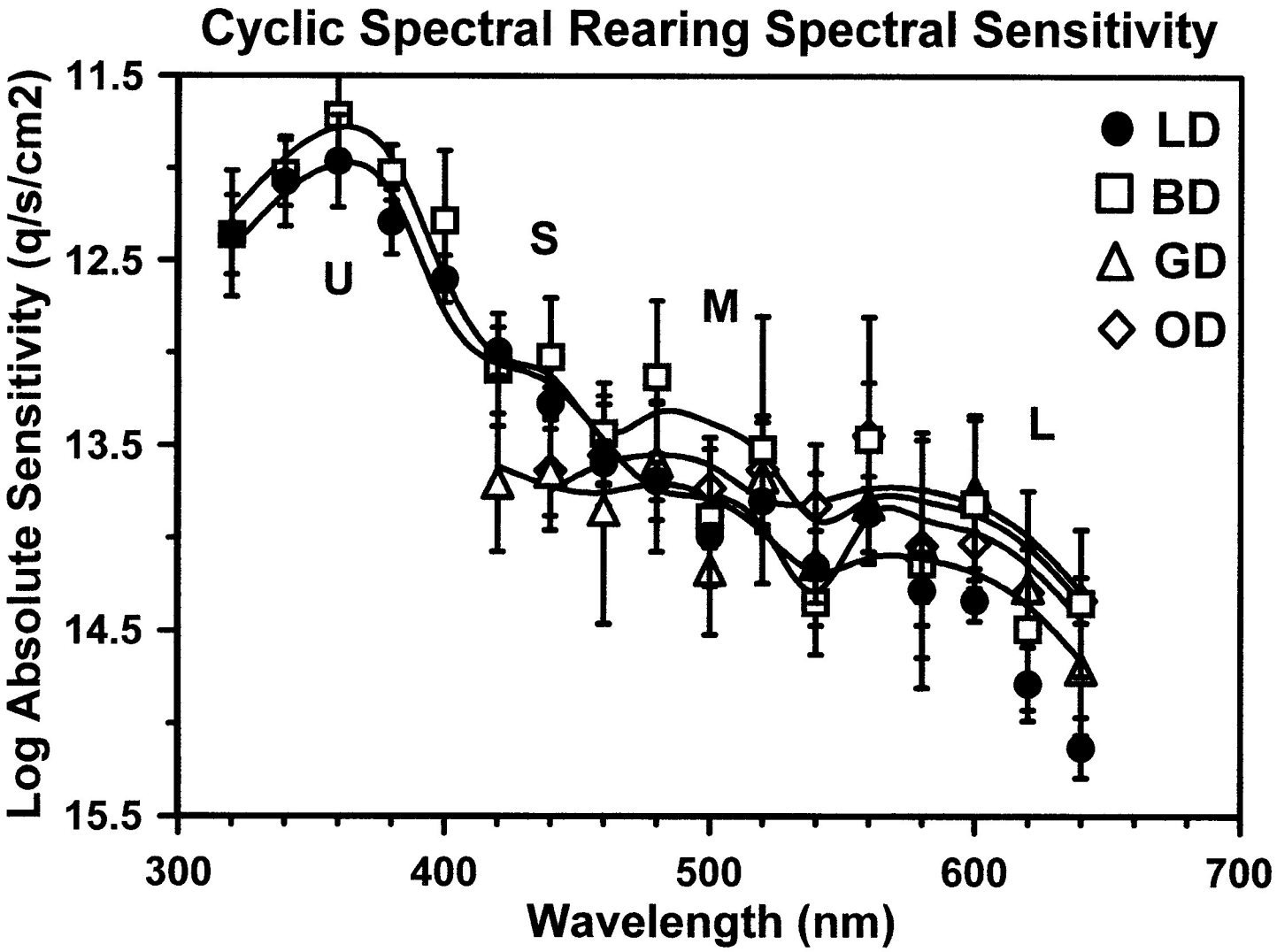

OO b-wave Spectral Sensitivity
Log Absolute Sensitivity (q/s/cm2)
11.5
12.5
13.5
14.5
15.5
300
400
500
600
700
Wavelength (nm)
LD
OO
U
S
M
L

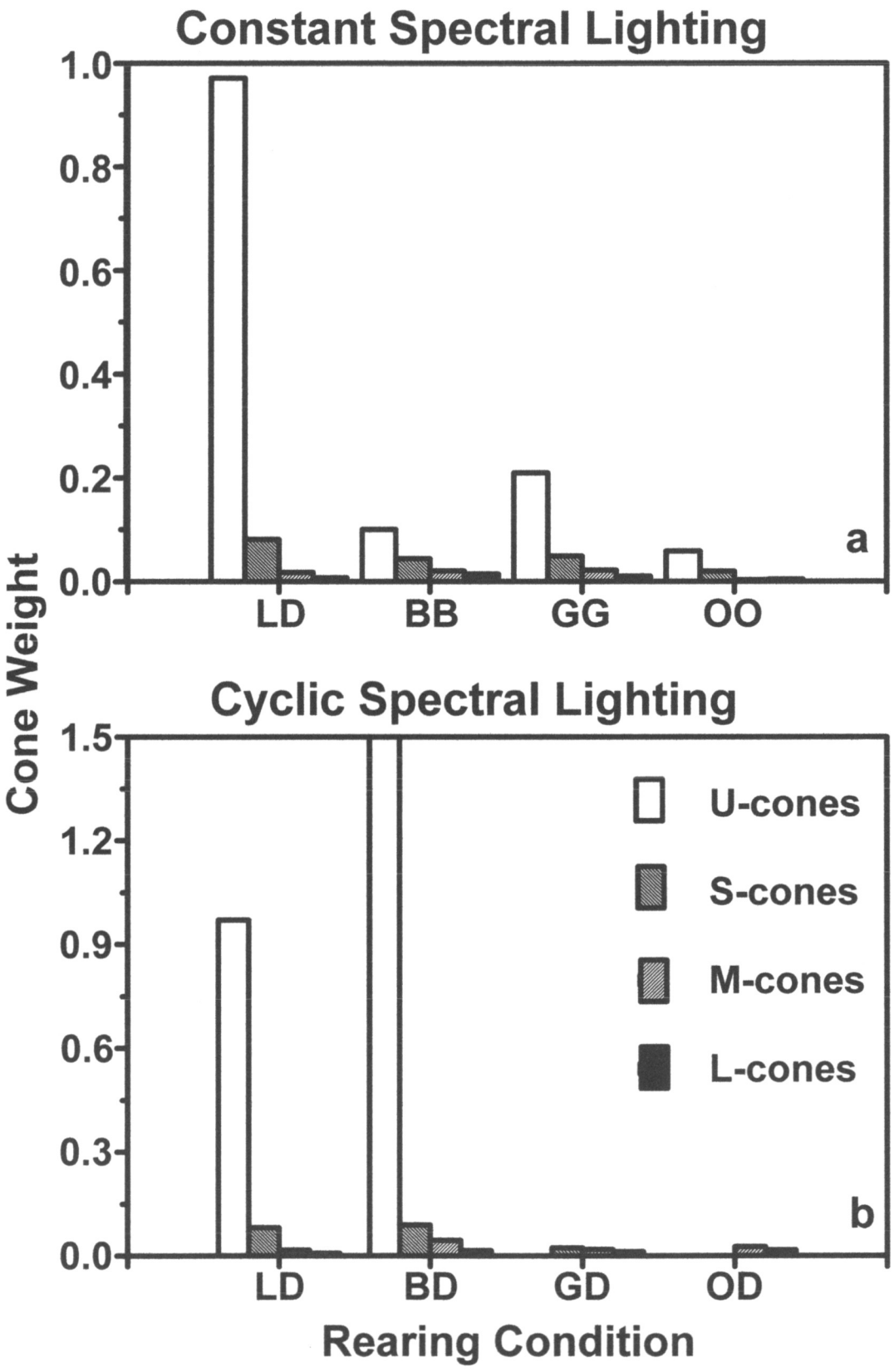

Constant Spectral Rearing Spectral Sensitivity
Log Absolute Sensitivity (q/s/cm2)
11.5
12.5
13.5
14.5
15.5
300
400
500
600
700
Wavelength (nm)
U
S
M
L
LD
BB
GG
OO


BD b-wave Spectral Sensitivity
Log Absolute Sensitivity (q/s/cm2)
11.5
12.5
13.5
14.5
15.5
300
400
500
600
700
Wavelength (nm)
LD
BD
U
S
M
L


GD Spectral Sensitivity
Log Absolute Sensitivity (q/s/cm2)
11.5
12.5
13.5
14.5
15.5
300
400
500
600
700
Wavelength (nm)
U
S
M
L
LD (B)
GD (B)
GD(A)


OD Spectral Sensitivity
Log Absolute Sensitivity (q/s/cm2)
11.5
12.5
13.5
14.5
15.5
300
400
500
600
700
Wavelength (nm)
LD (B)
OD(B)
OD(A)
U
S
M
L


Cyclic Spectral Rearing Spectral Sensitivity
Log Absolute Sensitivity (q/s/cm2)
11.5
12.5
13.5
14.5
15.5
300
400
500
600
700
Wavelength (nm)
U
S
M
L
LD
BD
GD
OD


Constant Spectral Lighting
1.0
0.8
0.6
0.4
0.2
0.0
LD
BB
GG
OO
a
Cyclic Spectral Lighting
1.5
1.2
0.9
0.6
0.3
0.0
LD
BD
GD
OD
b
U-cones
S-cones
M-cones
L-cones
Cone Weight
Rearing Condition